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Time-dependent kinetics of verapamil in rats

by

Yao Peng ©

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Canada T6H 5A1

Date: 25 August 1999

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduated Studies and Research for acceptance, a thesis entitled "Time-dependent kinetics of verapamil in rats" submitted by Yao (Penny) Peng in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences (Pharmacokinetics).

Dr. Y. K. Tam, Supervisor

Coutts

Dr. R. T. Coutts

Fak. Jamali

Dr. F. Jamali

Dr. L. I. Wiebe

Dr. M. R. Gray

Venkat

Dr. R Venkataramanan, External Reader

Date: 25 August 1999

DEDICATION

To my husband, my son, and my parents.

ABSTRACT

The pharmacokinetics of verapamil (VER) is characterized by extensive inter- and intra-individual variability. Time-dependent reduction in VER clearance has been observed in humans after chronic oral administration. The mechanisms responsible for this observation are not well characterized. The objectives of the current project are to examine the potential source of variability and the mechanisms of time-dependency.

HPLC analysis revealed that the recovery of VER from aqueous solutions was decreased to about 50% when solution pH was increased from 2.2 to 8. Exhaustive solubility, stability and surface activity studies of VER showed a rapid and nonspecific adsorption of VER to commercial polypropylene pipette tips during sample processing. The addition of bovine serum albumin to aqueous solutions of VER eliminates the adsorption problem.

After repeated oral administration of VER (five doses of 10 mg/kg given every 8 hours) significant increases in AUC (36.8%), oral bioavailability (53.7%) and decrease in oral clearance (29.9%) of S-VER were observed in rats. The kinetic parameters for R-VER did not change significantly after multiple dosing. These results indicate that VER undergoes stereoselective time-dependent kinetics in the rat.

In isolated perfused rat liver system VER demonstrated dose and time-dependent hepatic extraction. The degree of saturable first-pass liver metabolism differed for VER enantiomers with S-VER reaching saturation faster than did R-VER. VER was extensively accumulated and strongly retained in liver tissue as denoted by a high binding capacity, a high partition co-efficient and long washout half-lives. Thus, the time-

dependent kinetics of VER in the rat was mainly due to a combination of stereoselective enzyme saturation and extensive hepatic tissue binding. Enzyme inactivation probably plays a minor role.

The potential effects of lidocaine, diltiazem, diphenhydramine and norverapamil on the metabolism of VER were examined using freshly prepared isolated rat hepatocytes. All of these tertiary amines were capable of decreasing the metabolism of VER when each of them was co-incubated with VER, suggesting that common isozymes are involved in their disposition. Since the apparent K_i values of these drugs are relatively high compare to their therapeutic concentrations and the K_m of VER, it is predicted that these tertiary amines would not interact significantly with VER *in vivo* when given concomitantly. However, prolonging the exposure of hepatocytes to lidocaine, diltiazem and diphenhydramine significantly enhanced the inhibition of VER metabolism. These results imply that inhibition by these drugs is likely due to mechanism-based and long-term exposure to these tertiary amines which may promote interaction with VER. This study indicates that different prediction might be obtained, dependenting on the design of the *in vitro* experiment. It is important to identify the existence of mechanism-based inhibition as a mechanism involved in drug interactions.

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GLOSSARY OF ABBREVIATIONS AND SYMBOLS

α	probability of making a type I error
°C	degree Celsius
%	percent
Σ	summation
AAG	alpha-1-acid glycoprotein
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
AUC	area under the plasma concentration *vs.* time curve from time 0 to ∞
AUFC	area under the flux *vs.* time curve
AUMC	area under the first moment curve
Cb	VER concentration in whole blood
Cp	VER concentration in plasma
C_{in}	inlet concentration
C_{max}	peak drug concentration in plasma after an oral dose
C_{out}	outlet concentration
C_{ss}	steady-state concentration
Cl_H	hepatic clearance
Cl_o	oral clearance
Cl_s	systemic clearance
Cl_{int}	hepatic intrinsic clearance

CV	coefficient of variation
CYP	cytochrome P-450
D-617	2-(3,4-dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile
D-620	5-amino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile
DPH	diphenhydramine
DZ	diltiazem
dpm	disintegrations per minute
E	extraction ratio
EGTA	ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
F	absolute bioavailability
f_u	free fraction in plasma
G	gauge
g	gram(s)
g	gravity
h	hour(s)
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HPLC	high performance liquid chromatography
id	inside diameter
IU	international unit(s)
i.p.	intraperitoneal
I.S.	internal standard
i.v.	intravenous
HCl	hydrochloride

IPRL	isolated perfused rat liver
kg	kilogram(s)
K_i	inhibition constant
K_m	Michaelis Menten constant
K_{tw}	partition coefficient between the liver tissue *vs.* buffer solution in the sinusoid
L	litre(s)
LID	lidocaine
μg	microgram(s)
μL	microlitre(s)
μM	micromolar
μmoles	micromoles
M	molar
MI	metabolite-intermediate (complexes)
min	minute(s)
mg	milligram(s)
ml	milliliter(s)
n	number of observations
ng	nanogram(s)
NaOH	sodium hydroxide
nmol	nanomole(s)
NOR	2,8-bis (3,4-dimethoxyphenyl)-2-isopropyl-6-azaoctanitrile
P450	cytochrome P450

p	probability of rejecting the null hypothesis when it is true
PBS	phosphate buffered saline
p.o.	oral administration
pH	negative logarithm of concentration of H^+ ions
pK_a	negative logarithm of equilibrium constant of acid
ρ	density of the liver
Q	liver perfusion rate
r	correlation coefficient
r^2	squared correlation coefficient
R-VER	R-verapamil
SD	standard deviation
Sec	second(s)
S-VER	S-verapamil
τ	dosing interval
$t_{1/2}$	elimination half-life
TEA	triethylamine
T_{max}	time at which C_{max} is reached
T_{ss}	time to reach steady state during continuous drug infusion
U	unit(s)
UV	ultraviolet
v	rate of elimination
VER	verapamil (racemic of R and S isomers); 2,8-bis-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile

v/v	volume to volume ratio of mixtures
V_{dss}	volume of distribution at steady state
V_{max}	maximum velocity
W	weight of the liver
w/v	weight per volume

1. INTRODUCTION

1.1 Nonlinear pharmacokinetics

When the rate of drug elimination from the body at any time is proportional to the amount of drug in the body at that time, a first-order or linear process is operative. Thus, the term 'linear pharmacokinetics' indicates that for a given drug all concentration time profiles that are normalized for time and size of dose should be superimposible. The term 'nonlinear pharmacokinetics' implies such profiles are not superimposible, due to one or more time- or dose-related dependencies (*Ludden et al. 1991*).

Causes of nonlinear behavior can be divided into dose or concentration-related factors and time-dependent factors. Reasons for lack of superposition include changes in pharmacokinetic parameters with size of dose or dosing rate; the pharmacokinetics of such drugs is said to be dose-dependent. When there is a lack of superposition on administering the same dose of a drug on separate occasions or a lack of predictability following repeated or continuous dosing, based on single-dose data, the drug is said to show time-dependent kinetics. These nonlinear behaviors are sources of variability in drug response. Albeit relatively uncommon, this phenomenon occurs frequently enough in drug therapy to warrant special consideration.

1.1.1 Concentration-dependent kinetics

Nonlinear processes may operate with respect to the absorption, distribution, metabolism and excretion of drugs. Nonlinearity in absorption can lead to an increase in drug concentration that is disproportionately to an increase in dose. Change in bioavaibability may be due to poor solubility in luminal fluid at higher doses, which is observed with griseofulvin (*Barrett et al. 1975*), saturation of a carrier system such as seen with amoxycillin (*Svjoall et al. 1985*), and saturation of metabolic processes responsible for presystemic metabolism such as with salicylamide (Siddoway *et al. 1967*). Saturable first-pass metabolism occurs for a number of orally administered drugs that are highly extracted by the liver or intestinal tissues. In such cases, circulating drug concentrations are sensitive not only to dose but also to rate of absorption. Examples of drugs that appear to exhibit a significant saturable first-pass effect following oral administration include propafenone (*Siddoway et al. 1967*), propranolol (*Siddoway et al. 1987*), hydralazine, imipramine (*Silber et al. 1983*) and paroxetine (*Shepherd et al. 1984*).

The binding of drug to plasma constituents, blood cells and extravascular tissue may exhibit concentration dependence. This can cause pharmacokinetic parameters based on total blood or serum drug concentrations to be concentration-dependent. For drugs that show saturable binding to plasma proteins, the volume of distribution is expected to increase with plasma concentration (*Øie and Tozer 1979*). Examples of drugs in this category include valproic acid (*Sheyer et al. 1990*) and disopyramide (*Hinderling and Garrett 1976)*, which exhibit concentration-dependent plasma binding near or within the usual therapeutic range. Conversely, for drugs that show saturable tissue binding such as S-warfarin (*Cheung et al. 1989*) and quinidine (*Harashima et al. 1985*), the volume of distribution decreases as plasma concentration is increased. However, the effect of tissue binding on volume of distribution may be complicated by the likelihood that not all tissues are saturated to the same extent. Depending on the degree of non-linear tissue binding, the volume of distribution of a drug may not be sensitive to the changes in tissue binding (*Chen and Gross 1979*).

Saturation or partial saturation of one or more pathways for the systemic elimination of a drug is one of the major causes of nonlinear elimination. The clearance of drugs in the liver depends on the affinity of the drug for metabolic enzymes, expressed as the Michaelis-Menten constant (K_m) and the capacity of these enzymes (V_{max}). Nonlinear pharmacokinetic behavior will be most evident when concentrations are close to or above a K_m value or the rate of administration required to achieve these concentration approaches V_{max}. The classic example of a drug that exhibits Michaelis-Menten pharmacokinetics is phenytoin. This drug has therapeutic serum concentrations that are (10-20 mg/L) 2 to 4 times the mean K_m value (5.6 mg/L), thus a disproportionate increase in concentration is observed at, and above, the therapeutic concentration range (*Bauer and Blouin 1983*). In some cases, the nonlinear elimination characteristics could not be readily rationalized by simple Michaelis-Menten kinetics. Metabolites of a drug may cause inhibition of the metabolism of parent drug such as phenylbutazone (*Jahnchen et al. 1972*) and nicorandil (*Bachert et al. 1994*). Saturable renal tubular secretion rarely results in clinically important nonlinear plasma pharmacokinetics. For saturable secretion to have a significant effect on the plasma concentration-dose-time relationship, the pathway must account for at least 20 to 30% of drug clearance and drug concentrations must equal or be greater than the apparent K_m of the system (*van Ginneken and Russel 1989*).

1.1.2 Time-dependent kinetics

Time-dependent kinetics, unlike dose-dependent kinetics, involves an alteration in the biochemistry in an organ or physiologic change in the patient (*Levy et al. 1982*). One type of time dependency is termed *chronopharmacokinetics*, which describes changes in pharmacokinetic parameter due to normal physiologic circadian rhythms. A second type of time dependency leading to nonlinear pharmacokinetics is based on a biochemical change mainly due to auto-induction or auto-inhibition of metabolic enzymes. For example, upon repeated dosing, carbamazepine induces enzymes responsible for its clearance (auto-induction), thereby increasing the elimination of the drug (*Pitlick et al. 1977*). Well-documented examples of auto-inhibition are the macrolide antibiotics, troleandomycin and erythromycin. Cytochrome P-450 (CYP) enzymes convert these drugs to reactive metabolites that bind within the catalytic center of the enzyme (*Larrey et al. 1983*). Production of such reactive metabolites can lead to covalent modification of the CYP isozymes and irreversible loss of oxidative function. This process gives rise to long-term effects on pharmacokinetics as the inactivated CYP isozymes must be replaced by newly synthesized CYP protein (*Murray et al. 1997*).

The literature on time-dependent kinetics is not extensive compared to dose dependent kinetics, which might be due to the fact that studies of time dependency involve longitudinal observations beyond those of typical single dose pharmacokinetic studies. A time-dependent phenomenon involves actual physiological or biochemical changes in the organ(s) of the body associated with the drug disposition parameters. It could be distinguished from concentration- or dose-dependency by the fact that pharmacokinetic parameters are altered with time while drug concentration or dose is invariant. However, it is possible that time dependency originates from an avid liver tissue binding or coexistence of liver tissue binding and dose dependency.

1.1.2.1 The effect of tissue binding on time-dependent kinetics

The effect of liver tissue binding on time-dependent kinetics has not been extensively explored. Using a two-compartment system consisting of a reservoir and a liver, it has been demonstrated mathematically that the average clearance of a drug after a single bolus dose is not influenced by tissue binding when elimination is first-order (*Jusko and Gibaldi 1972, Rowland et al. 1973, Wedlund and Wilkinson 1984*). However, Wedlund (*1984*) also has shown that the degree of tissue binding influences the time course of a drug in the systemic circulation after a single oral dose. It is characterized by a reduced peak plasma concentration and prolonged elimination half-life. The last phase of drug elimination may not be able to be detected with common analytical methodology and causes a so-called "threshold dose" phenomenon, exemplified by the kinetics of propranolol in human (*Evans and Shand 1973*), rat (*Evans et al. 1973*), and dog (*Bai and Abramson 1984*).

In addition, it has been speculated that if after loading of the liver with a drug which is slowly released from binding sites or diffused back into plasma that a disequilibrium between the unbound concentration in liver and plasma exists, total first-pass extraction after initial dose would be greater than that after multiple dose (*Thummel et al. 1997*). Furthermore, if a high portal venous concentration can saturate the hepatic binding sites after a single oral dose and the high-affinity site remained saturated during repetitive dosing, subsequent oral doses would be less well extracted resulting in unexpected drug accumulation (*Evans et al. 1973*). There is evidence that reversible and irreversible binding in the liver are responsible for time-related changes in clearance of propranolol (*Evans et al. 1973*) and diltiazem (*Hussain et al. 1994*).

1.1.2.2 Coexistence of extensive liver tissue binding and saturable metabolism

The effect of hepatic tissue binding on drug metabolism when the metabolism is saturable (i.e. obeys Mechaelis-Menten kinetics) was simulated by Rubin and Tozer (*1986*). It has been demonstrated that when a drug exhibits a low K_m and extensive tissue binding, the uptake of drug to hepatic binding sites during first-pass will tend to reduce the likelihood of enzyme saturation. Simulations show that during the initial distribution phase after a single dose, hepatic binding sites act as a "sink" and thereby compete with the metabolizing enzymes. After this phase is completed, net dissociation of drug occurs and those sites that are bound act as a source of drug for the enzymes, thus increasing the tendency to saturable metabolism. After loading the liver with a drug, which is extensively bound to liver tissue, the hepatic extraction after initial dose might be greater than that after repetitive dose, because there is a slow release of bound drug in the liver. This scenario might apply to VER, which partitions extensively into the liver during first-pass and exhibits low oral bioavailability.

1.2 Verapamil

VER [2,8-bis-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile], an analogue of papaverine, was the first L-type calcium antagonist introduced into therapy in the early 1960s (Figure 1.1). With an aliphatic nitrogen, VER is a weak base and exists predominantly in the protonated form in physiological media with a pK_a of 8.9

(*Surakitbanharn et at. 1995*). This is a highly lipophilic drug with an octanol-water coefficient (log P) of 3.79 for the free base (*Carmen et al. 1992*).

The single chiral center in the chemical structure of VER (see p. 32) results in two stereoisomers: S(-)-verapamil (S-VER) and R(+)-verapamil (R-VER). Currently, all marketed verapamil products are racemic mixtures of the two enantiomers.

1.2.1 Pharmacology

1.2.1.1 Therapeutic usage

VER inhibits voltage-dependent L-type calcium-channels and is classified as a class IV anti-arrhythmic agent. Like all calcium channel blockers, VER modulates ionic calcium influx across membranes of conductile and contractile myocardial cells, as well as arterial smooth muscle. Calcium plays an important role in the excitation-contraction coupling process of the heart and vascular smooth muscle cells and in the electrical discharge of the specialized conduction cells of the heart (*Katz 1992*). The modulation of calcium influx slows atrioventricular conduction, reduces myocardial contractility and systemic vascular resistance, and results in coronary and peripheral vasodilation. VER is currently indicated for controlling angina, hypertension, paroxysmal supraventricular tachycardia, and rapid ventricular atrial flutter or fibrillation (*Dustan et al. 1989, McTavish et al. 1989, Echizen et al. 1986, Chatterjee et al. 1984, McAllister et al. 1982*). VER is available as conventional or sustained release formulations for oral administration. For the treatment of essential hypertension, the recommended starting dose of oral VER tablets in conventional formulation is 80 mg 3 times daily and 240 mg once daily for sustained release formulations (*McTavish and Sorkin 1989*). In patients with stable angina pectoris, plasma concentration in the range of 150 to 500 ng/ml has been associated with an increased maximal work capacity (*Weiner et al. 1989*). In patients with hypertrophic obstructive cardiomyopathy, VER plasma concentrations ranging from 100 to 400 ng/ml were associated with clinical improvement during long term administration (*Woodcock et al. 1980*). However, the intra- and inter-subject variability in intensity of drug effect seen at specific plasma drug concentrations has made definition of a 'therapeutic' range difficult. Routine therapeutic drug monitoring is recommended because of broad therapeutic and toxic potential (*Haman et al.1984*) and the large intra- and inter-individual variabilities in its disposition kinetics during long term dosing (*Eichelbaum et al. 1984, Harder et al. 1991*).

1.2.1.2 Adverse reactions

Treatment with VER is generally well tolerated, but adverse effects connected with its pharmacological effects on cardiac conduction can arise and may be particularly severe in patients with hypertrophic cardiomyopathies. The common adverse effects are constipation and other non-cardiac adverse effects including dizziness, fatigue, nausea, peripheral edema and headache. Adverse effects on the heart include palpitation, bradycardia and orthostatic hypotension (*Bala Subramnian et al. 1981, McTavish et al. 1989*). Severe hypotension (*Buxton et al 1987*), first and second degree heart block (*Prystowsky et al. 1988*), ventricular fibrillation (*McGovern et al. 1986*), life-threatening bradycardia, cardiorespiratory collapse (*Epstein et al. 1985*), rebound angina (*Raftos et al. 1980*) and conduction disturbances (*Bala Subramnian et al. 1981*) have been reported.

1.2.2 Pharmacokinetics

1.2.2.1 Absorption

VER is almost completely absorbed since no differences were observed in the cumulative urinary excretion of ^{14}C activity and the area under the plasma concentration-time curve (AUC) of ^{14}C activity between the intravenous and oral route of administration of same dose of the drug. Less than 5% of the dose was excreted in the urine as unchanged drug (*Eichelbaum, et al. 1981*). This radioactivity study does not provide absolute proof that VER is absorbed completely intact across the gastrointestinal (GI) tract because it does not rule out the possibility that VER undergoes first pass GI tract metabolism. The complete absorption of VER in rats has been shown in the study by Hoffman *et al* (*1995*). Using an absorption probe and portal blood sampling method, it was found that VER absorption was rapid and complete within 1 hour as evidenced by the merging of the portal and systemic concentration-time profiles. Due to extensive first-pass hepatic extraction (E: 0.8-0.9), the bioavailability of VER in various animal species is typically 10-20% (*Schomerus et al. 1976*). Average time to peak plasma concentration is between 1-2 hours after ingestion of the drug. After oral administration of a 120-mg dose of VER every six hours, plasma levels range from 125-400 ng/ml (*Guerrero et al. 1984*). The presence of food in the gastrointestine seems to have no effect on the bioavailability of VER, but can prolong its T_{max} (*Woodcock et al. 1986*). Patients with liver disease have a significantly increased bioavailability (*Woodcock et al. 1981*).

1.2.2.2 Distribution

VER is widely distributed throughout the body with a mean apparent volume of distribution in volunteers ranging from about 113 to 418L after intravenous administration (*Anderson et al. 1986 and 1982, Somogyi et al. 1981*). The distribution of VER in rats after a single oral dose is predominantly in the liver, where 70% of the drug was recovered between 15 to 30 min after dosing. Almost half of the administered VER was found in the lungs and only 17% in the livers after IV infusion (*Hamann et al. 1983).* In the study by Todd *et al*. (*1987*) using female rats, VER has been shown to extensively distribute into tissues such as lung, kidney, and liver. Although lipophilicity has been shown to be an important determinant of its tissue distribution, Todd's work suggests that other physicochemical and biological properties are also crucial. VER is highly bound to plasma protein in man. The fraction unbound is in the order of 8.7 to 16% (*McGowan et al. 1983*). Like other basic drugs, VER is mainly bound to α_1-acid glycoprotein and albumin and the concentration of α_1-acid glycoprotein appeared to be the major contributory factor in individual variation in protein binding (*McGowan et al. 1983*). It has been reported that VER can be displaced from its plasma binding sites *in vitro* by certain basic drugs, e.g. lidocaine, diazepam, propranolol and disopyramide (*Yong et al. 1980*).

1.2.2.3 Elimination

VER undergoes extensive metabolism in humans and animals. However, only 55% of the metabolized drug has been accounted for in terms of identifiable metabolites (*Nelson et al. 1988a and 1988b*). The complex pattern of VER oxidative metabolism has been extensively studied. A schematic diagram of metabolic pathways is depicted in Figure 1.1. The main metabolic pathways include N-dealkylation and O-demethylation. N-dealkylation, is the primary route of metabolism for VER and occurs more rapidly than O-demethylation by about 2-fold and accounts for about 30-40% of all known metabolites (*Nelson et al. 1988b*). N-dealkylation leads to at least 12 metabolites, of which three are major ones: Norverapamil (NOR) , D617 and D620. The total amount of those metabolites excreted in urine are about 6% (NOR), 22% (D617) and 4% (D620) (*Eichelbaum et al. 1978*) . Studies with antibodies and with individual human CYP isozymes have demonstrated CYP3A4 and CYP1A2 to be the enzymes responsible for VER N-dealkylation, whereas enzymes of the CYP2C subfamily are mainly involved in VER O-demethylation. This pathway results in the formation of D-703 and D-702. CYP2D6 is not involved in N-dealkylation, N-demethylation, or O-demethylation (*Kroemer et al. 1992 and 1993, Busse et al. 1995*).

NOR has been found after oral VER administration and has approximately 20% of the coronary vasodilation activity of the parent compound (*Neugebauer et al. 1978*). Although O-demethylated metabolites of VER exhibits the same potency as the parent drug, their contribution to the overall pharmacological effect is negligible since these metabolites are present in plasma as glucuronides which lack pharmacological activity (*Woodcock et al. 1981,Hamann et al. 1984).*

The liver is the major site of biotransformation and first-pass metabolism of VER and gut metabolism of VER is not considered to be important. This is based on the observation of a large increase in absolute bioavailability (38% to 81.5%) in cirrhotic patient in whom the blood flow to the liver was bypassed by a mesocaval shunt surgery

(*Eichelbaum et al. 1980*). Also, in patients with liver diseases the clearance of VER was reduced by almost 60% (*Woodcock et al. 1981*). However, it has been shown recently that orally administered VER is probably metabolised to an appreciable extent (approximately 30%) in the intestinal mucosa (*Fromm et al. 1998*). Mooy *et al* (*1985*) demonstrated that the disposition of verapamil was similar in normal subjects and in patients with renal failure.

1.2.2.4 Excretion

VER is mainly excreted as metabolites in humans. Approximately 70% of the metabolites are excreted in the urine and 16% in the feces (*Eichelbaum et al.1978*). Only about 3-4% is excreted as unchanged drug in the urine and renal elimination is marginal even at high doses (*Toffoli et al. 1997*). The conjugation products of O-demethylated metabolites are preferentially secreted into the gut via the biliary route and a minor amount of VER is present in rat's bile (*McIlhernny et al. 1971*).

1.3 Variability

VER is well known to demonstrate high inter- and intra-variabilities in its pharmacokinetics. By using stable isotope techniques, it was shown that the clearance of VER during chronic oral administration exhibited considerable inter- and intra-individual variation, ranging between 26.3% to 85.4%, and 12.0% to 48.0%, respectively (*Eichelaum and Somogyi 1984*). Tsang *et al.* (*1996*) evaluated the bioavailability of VER in healthy volunteers given the same dose on two occasions. The AUC values varied 3.8-fold and the C_{max} varied more than 9-fold among subjects. The intra-subject difference between days ranged from –46% to +298% for C_{max}. For VER sustained release preparations, the intra-subject variability for C_{max} increased from 26% for a single dose to 31% for multiple doses (*Ahmed et al. 1995*). The variability of several important pharmacokinetic parameters (C_{max}, T_{max} and $T_{1/2}$) of VER was significantly reduced by sublingual application in comparison to the oral route, indicating that the major source of the variability was from the first pass metabolism (*Woodcock et al 1982*).

It has been a long accepted notion and has been demonstrated by Hellriegel *et al.* (*1996*) that there will be greater inter-subject variability in oral drug bioavailability when a drug exhibits a high degree of first-pass metabolic extraction, than when first-pass extraction is low. Factors such as age (*Abernethy et al. 1986 and Sasaki M. 1993*), disease states (*Somogyi et al. 1981*), time of dosing (*Hla et al. 1992 and Gupta et al. 1996*) have been implicated in the observed variability in the pharmacokinetics of VER. In addition, the presence of non-linear kinetics, stereoselectivity, active metabolites and the effect of concomitant drugs might offer further explanations for the considerable inter- and intra-subject variabilities. Variable oral bioavailability will have its most serious impact for drugs with a narrow therapeutic range. The source of this constitutive variability is still largely unknown. Therefore, an understanding of the source of variability is the key to the optimization of VER therapy.

1.4 Stereoselectivity

VER is a racemic mixture with R- and S- enantiomers. These enantiomers have different pharmacokinetic and pharmacodynamic properties. In humans, the plasma clearance of S-VER is about twice that of R-VER and this higher clearance makes the bioavailability of S-VER correspondingly lower (*Eichelbaum et al. 1984*). The free fraction of S-VER is about twice that of R-VER. This difference in the free fraction contributes to the volume of distribution of S-VER being about twice that of R-VER (6.4 L/kg *vs.* 2.7 L/kg), and the plasma clearance of S-VER being about twice that of R-VER (18 ml/min/kg *vs.* 10 ml/min/kg) (*Gross et al. 1988).* Since the hepatic clearance of the S-VER is greater because of stereoselective first pass metabolism, the R/S ratio of VER after oral administration is substantially higher, 4 to 5 times, than after single intravenous administration (*Vogelgesang et al. 1984*).

Several investigators have attempted to identify the metabolic pathway of VER responsible for the stereoselectivity. The biochemical behavior that is responsible for this substantial stereoselectivity in humans has not been established. Nelson and co-workers (*1988a* and *1988b*) examined the stereospecificity of VER O-demethylation and N-dealkylation by human liver microsomes. However, they could not identify a metabolic reaction with sufficient stereoselectivity to account for the ratio observed in humans. Also, Kroemer *et al.* (*1993*) found no pronounced stereoselectivity in the formation of metabolites D-617 and NOR. Cashman (*1989*), however, in a study involving rat and dog liver microsomes and purified flavin-linked monooxygenase (FMO) from both species, discovered an unusual and highly stereospecific dealkylation reaction. Ayesh *et al.* (*1991*) could not substantiate this finding in humans and noted no difference in the metabolic pattern of VER in patient with an inherited deficiency of FMO in the N-oxidation of trimethylamine. It is possible that the O-demethylation pathway predominantly contributes to the stereoselectivity in humans (*Mikus et al. 1990*).

Although both enantiomers have similar types of pharmacological activity, the S-enantiomer has been shown to be more pharmacologically active than its antipode in the cardiovascular system. For example, S-VER is 10-20 times more potent in terms of negative dromotropic effect on atrioventricular (AV) conduction (*Echizen et al. 1986*); 5-15 times for negative inotropic and negative chronotropic effects and 2.5 times greater vasodilation potency than R-VER (*Satoh et al. 1980*). However, not all pharmacological activities differ for S- and R-VER. For instance, the two enantiomers have nearly identical activities in overcoming multiple-drug resistance to several oncologic agents in drug-resistant cell lines (*Plumb et al.1990*).

The actual value of the enantiomeric ratio (R/S) in plasma is determined by a combination of factors. Pharmacokinetic distinctions between the two enantiomers (e.g., volumes of distribution, clearance, protein binding) are the ultimate source of differences, but external factors such as the route of administration and the rate of absorption are also critical.

1.5 Nonlinear kinetics of verapamil

Nonlinear pharmacokinetics of VER in humans has been observed since 1981 and its origin remains somewhat controversial. Hepatic metabolism of VER depends on the route (oral or i.v.), the rate of oral input (immediate or sustained release), and the schedule of administration (single *vs.* repeated doses). Kates *et al.* (*1981*) utilized model-independent parameters generated from single-dose intravenous data to effectively predict steady-state plasma concentrations following a multiple-infusion scheme designed to achieve plasma drug concentrations over a range of 19.6 to 118.5 ng/ml. This study suggested linear pharmacokinetics following intravenous therapy. In contrast, Bourne *et al.* (*1980*) found that a pharmacokinetic model derived from single intravenous doses in normal subjects allowed rapid achievement of predicted plasma VER concentrations and maintenance during continuous infusion. However, it was only accurate for drug levels below 60 ng/ml and progressively underestimated drug concentrations above this level. Non-linear accumulation of VER has been observed following multiple oral doses. Compared with a single oral dose, a significant increase in bioavailability and a decrease in oral clearance have been reported with multiple VER doses (*Eichelbaum et al. 1981 and 1984, Shand et al. 1981, Schwardz et al. 1982, Meredith et al. 1985, Freedman et al. 1981, Rumiantsev et al. 1988.*). The observed steady-state plasma concentrations were, on average, 2 to 3 times higher than the predicted steady-state concentrations using pharmacokinetic data obtained from single oral dose studies, suggesting either an increased systemic bioavailability or decreased systemic clearance. No time dependency, but dose dependency was observed by Harder et al. (*1991*).

The question of whether or not there is an increase in VER half-life following multiple dosing remains unanswered. In contrast to several reports documenting substantial increase in VER half-life (*Freedman et al. 1981, Schwartz et al. 1982 and 1985, Shand et al. 1981, Rumiantsev et al. 1988*), no increase in elimination half-life was observed by Eichelbaum and Somogyi (*1984*) during long term dosing. Nevertheless, as a result of chronic dosing higher drug concentrations may permit better characterization of the terminal elimination phase. This may explain the apparent increase in half-life after multiple dosing (*Echizen et al. 1986*).

VER has a high hepatic extraction ratio and some or all factors involved in the extent of hepatic first pass extraction including hepatic blood flow, plasma protein binding and intrinsic clearance of free drug, have been examined in humans. Despite numerous efforts in the past years, the accumulation of plasma VER during long term administration has not been fully elucidated. An understanding of the causes of nonlinearity and the implications of such behavior on concentration-time profile is required if such drugs are to be used safely and efficaciously.

1.5.1 Hepatic blood flow

VER is a high extraction drug and its clearance is blood flow rate-limited. Since VER is a vasodilator, any change in hepatic blood flow rate will change the clearance of the drug. A good correlation between VER clearance and hepatic blood flow has been shown in the study by Woodcock *et al.* (*1981*). By measuring the hepatic blood flow rate at the end of a dosing interval, Schwartz *et al.* (*1985*) found no evidence of a change in hepatic blood flow during chronic drug administration compared with that before the start of treatment. However, a significantly higher hepatic blood flow rate after the first dose than at the steady-state dose was found when hepatic blood flow was measured at 1 hour after dosing (*Meredith et al. 1985*). An increase of hepatic blood flow has also been found by *Bauer et al.* (*1986*). It was concluded that this transient increase in blood flow rate could cause an increase in the clearance of VER, thereby lowering AUC during acute VER treatment (*Meredith et al. 1985*). Unfortunately, this argument is invalid. It is well documented in the literature that a transient increase in blood flow rate would increase the bioavailability of the drug. According to well stirred model, dependent upon the duration of increase, the net effect of increasing bioavailability and clearance would result in either an increase or no change in AUC (*Mclean et al. 1978, Nation et al. 1977*). Pang and Rowland (1977) have drawn similar conclusion based on the venous equilibrium model for hepatic clearance.

1.5.2 Plasma protein binding

In human VER was approximately 90% bound to plasma proteins and binding was independent of plasma concentration over a range of 35 ng/ml to 1557 ng/ml (*Keefe et al. 1981*). In addition, the metabolites D617 and D620 in concentrations up to 5000 ng/ml had no effect on the protein binding of R- and S-VER. *In vitro* results showed that NOR had no effect on protein binding of R- and S-VER up to a concentration of 1000 ng/ml. When NOR concentration was increased to 5000 ng/ml, a 30% increase in free fraction of both R- and S-VER was observed (*Johnson et al. 1995*).

Alteration of VER plasma protein binding will not significantly affect its hepatic clearance, since it is highly extracted by the liver and is considered to be flow limited. Oral bioavailability, on the other hand, would be sensitive to a change in plasma protein binding for the high extraction ratio drug. For example, the stereoselectivity in hepatic availability of VER in isolated perfused rat liver is significantly influenced by the stereoselectivity in the protein binding of the drug (*Mehvar and Reynolds, 1996*). The significant increase in VER bioavailability after multiple dosing could be caused by an increase in plasma protein binding. However, no change in protein binding of R- and S-VER following single and multiple doses of racemic VER was observed (*Johnson et al. 1995*). Thus, alterations in plasma protein binding could not be responsible for the time-dependent kinetics of VER.

1.5.3 Metabolic clearance

VER exhibits behavior that appears to be consistent with either Michaelis-Menten pharmacokinetics or “the altered enzyme hypothesis”, because a doubling of the dose (80 mg *vs.* 160 mg) causes apparent CL to decrease by about 25% and chronic treatment with this drug causes a decrease in CL value by about 50% (*Eichelbaum et al. 1984*). The mechanisms contributing to these nonlinear pharmacokinetic behaviors have been ascribed to a reduction in hepatic clearance associated with the saturation of metabolic processes (*Eichelbaum et al. 1981, Wagner et al. 1984, Woodcock et al. 1981, Schwartz et al. 1985*); a reduction in presystemic metabolism and thereby an increased systemic bioavailability (*Schwartz et al. 1985, Rumiantsev et al. 1986, Meredith et al. 1985*); or by the presence of a deep tissue compartment of drug distribution which is not detected after single doses (*Schwartz et al. 1985*). However, these postulations have not yet been evaluated to see if they can explain the time-dependent kinetics of VER.

1.5.3.1 Saturation in hepatic metabolism

Wagner (*1984*) has expressed a view that essentially all drugs that exert a significant first pass effect will exhibit non-linear Michaelis-Menten kinetics after therapeutic oral dosing. This is a result of high drug concentrations entering the liver during absorption. In this study, a predictable decrease in oral VER clearance following multiple-dose using typical Michaelis-Menten kinetics (K_m: 133 ± 43.3 ng/ml, V_{max}: 575 ± 10.7 mg/day) has been demonstrated. In addition, VER showed input rate-dependent stereoselective kinetics in both human (*Karim and Piergies 1995*) and rat (*Mehvar et al. 1994*), which suggest Michaelis-Menten type model may be operative in these species. From studies with slow release VER, Woodcock *et al.* (*1988*) compared data published by several authors and presented evidence for Michaelis-Menten first pass liver metabolism. At equal dosing rates, the slow release formulation had a significantly lower AUC and bioavailability when compared to the one with a rapid release. Similar results have been reported by Harder *et al.* (*1991*). Karim *et al.* (*1995*) reported significantly different R/S enantiomeric ratios of VER in the plasma with oral formulations that had substantially different rates of input. With the immediate-release formulation the total VER C_{max} was higher than that observed with the sustained-release formulation, and S-VER as percentage of the total VER was higher. These findings were attributed to the concentration- and/or input-rate related saturable hepatic first-pass metabolism of S-VER (*Karim et al. 1995*). A dose dependent change has also been shown after acute oral overdoses. It was suggested that saturable clearance might cause higher bioavailability and slower elimination of VER (*Toffoli et al. 1997*).

In a study by Gupta *et al.* (*1996*), the relationship between dose and plasma concentration of VER controlled-release in doses of 120, 180, 360, and 540-mg were examined. A dose-proportional relationship was found at both low VER input rates (120-mg and 180-mg) and high input rates (360-mg and 540-mg). However, nonlinearity was found between the 180-mg dose and 360-mg dose. Results from this study are consistent with the proposal that VER may have simultaneous first-order and capacity-limited first-pass extraction process (*Tsuchiya et al. 1972*).

However, there are studies that show that oral clearance and systemic bioavailability values following single oral administration is dose independent. A linear relationship was observed between dose and AUC following single oral administration of 80, 120 and 160 mg of VER to normal subjects (*McAllister et al. 1982, Frishman et al. 1982, and Anderson et al. 1982*). No marked saturation of VER elimination has been found in patients with acute poisoning of VER (*Kivisto et al. 1997*). Hence, the contribution of saturable metabolism to the nonlinear kinetics of VER needs to be further evaluated.

1.5.3.2 Product inhibition

A possible mechanism for nonlinear kinetics is product inhibition of drug metabolism, which can lead to time-dependent changes in the elimination. It has been found that nordiazepam inhibits the metabolism of diazepam (*Klotz et al. 1976*). Following multiple dosing a time-dependent reduction of diazepam elimination has been observed (*Klotz and Reimann, 1981*).

NOR was not found in plasma after iv administration but the concentration of NOR was comparable with that of VER after oral administration of VER (*Neugebauer et al. 1978*). The higher concentration of VER in the portal vein associated with oral VER, resulted in the production of a higher concentration of NOR (*Shand et al. 1981*). Although NOR accounted for only 10-20% of all metabolites, it was the only metabolite measurable in plasma after oral VER administration. It was found that the disappearance of NOR was slower than that of VER (*Frishman et al. 1982*). Oral dose studies by Kates *et al.* (*1981*) demonstrated significant accumulation of this active metabolite. However, it is not known whether NOR would inhibit the elimination of VER and whether the formation of NOR will contribute to the nonlinearity of VER kinetics.

1.5.3.3 Tissue binding in the liver

It was reported that VER is buried deeply in the hydrocarbon core of the biological membrane, intercalated between lipid acyl chains and acting as interstitial impurity (*Jorgensen et al. 1991*). Other information obtained by NMR measurements indicated a clear-cut substructure interacting with the phosphorous head group of membrane, where part of the molecule reached into the water phase (*Scheyer et al. 1990*). Extensive nonspecific binding of VER to lipid membrane was observed (*Erdreich et al.*

1987). Tissue binding of a lipophilic drug includes partitioning into lipid membranes and binding to proteins in the tissue, which can affect pharmacokinetics during the distribution phase. The liver plays a key role in VER metabolism, the distribution and binding of this drug to liver tissue can also be pharmacokinetically important.

There are evidences that reversible and irreversible binding in the liver is responsible for time-related changes in clearance of drugs. Hussain *et al*. (*1994*) observed that in an isolated liver perfusion system diltiazem (DZ) was taken up by the liver at 670-970 nmol/g liver tissue, which contributed to a long delay between infusion of the drug and the achievement of a constant effluent concentration. Liver models, which incorporated a tissue-binding component, were used to simulate time courses of lidocaine (LID) in a single-pass perfusion system (*Gray et al. 1987, Saville et al. 1986*). Results indicated that the effect of hepatic tissue binding was to delay the time to reach steady state and contribute partially to the time-dependency of LID. VER is more lipophilic compared to DZ and LID (*Scheufler et al. 1990*), and it is possible such a mechanism is responsible for the time-dependency of this drug.

The presence of a deep tissue compartment into which VER distributes that cannot be detected with current analytical methodology cannot be ruled out. During chronic dosing in which significant drug accumulation occurs, such a compartment would become identifiable by the terminal phase of drug elimination, and would result in prolongation of the observed $T_{1/2}$. A study by Gillespie *et al*. (*1984*) showed that isolated rat lungs sequestered but did not metabolize VER. The result of this was that VER persists in the lungs in the form of a non-effluxable pool which suggests that a deep tissue compartment exist.

1.5.3.4 Enzyme inactivation

Drugs with a secondary or tertiary amino-group such as *amphetamines (Franklin 1977*), erythromycin derivatives (*Larrey et al. 1983*), diphenhydramine analogs (*Bast et al. 1984, 1990*) can inactivate N-dealkylating CYP isozymes by the formation of a stable metabolic-intermediate complex with CYP isozymes (*Saville et al. 1989*). This phenomenon was postulated to be the main reason for the time-dependent kinetics of LID (*Tam et al. 1987*) and propranolol (*Weber et al. 1994*). VER undergoes extensive first-pass metabolism in the liver and the major phase I metabolic pathway is N-dealkylation in the human and in the rat (*Nelson et al. 1988*). It is possible that a similar mechanism of enzyme inactivation is responsible for the observed time-dependent kinetics of VER. VER is a potent inhibitor of drug metabolism both *in vitro* and *in vivo* (*Renton et al. 1985 and Bauer et al. 1986*). In humans, VER decreased the clearance of antipyrine (*Bauer et al. 1986, Rumiantsev et al. 1986 and 1988*). However, direct evidence of VER inactivating its own metabolism is lacking.

1.6 Drug interaction of verapamil

An important source of variability in drug metabolism is represented by inhibitory interactions between concomitantly administered drugs. Impairment of drug metabolism as a consequence of drug interaction has obvious clinical implications including toxicity. The resultant increase in concentration may be a result of increased bioavailability and decreased clearance. Kinetic variability can be magnified when inhibition leads to nonlinearity. Thus, understanding and anticipating pharmacokinetic studies of drug interactions have become a routine part of pharmaceutical drug development.

1.6.1 Competitive *vs.* mechanism-based inhibition

Competitive inhibition is the most common mechanism of inhibition of drug metabolism and occurs when two or more drugs compete for the same enzyme. The competitive process is likely to be concentration dependent. The reaction between enzyme and inhibitor is extremely rapid since no chemical reaction leading to a covalent bond formation is involved (*Meyer et al. 1996*). Therefore, *in vitro* coincubation of the substrate with an inhibitor should yield an estimate of the competitive inhibitory potency of the inhibitor.

However, it was recognized recently that the predictability of *in vivo* inhibition is reduced when drug interactions involve mechanism-based inhibition (*von Moltke et al. 1998, Benedett et al. 1998*). In most cases, mechanism-based inhibition is exhibited by its metabolites rather than by the parent compound. Unlike competitive inhibition, the generation of metabolites and the formation of stable metabolic intermediates (MI) take time and the maximum inhibitory effect on the enzyme is usually delayed. Production of such reactive metabolites can lead to covalent modification of the CYP and irreversible loss of oxidative function. New CYP must be synthesized to overcome the functional defect in drug oxidation. Hence, it is very important to identify the existence of such a mechanism involved in drug interaction.

1.6.2 The effect of some tertiary amines on the metabolism of verapamil

Many tertiary amines are therapeutically important agents; these drugs are extensively biotransformed in the liver. Drug interactions between these tertiary amines are well documented (*Hermann et al. 1992, Leinonen et al. 1991, Hussain et al. 1994, Amsterdam and Maislin 1991*). CYP 3A isozymes, often CYP 3A4 in human, are the most common isozymes responsible for N-dealkylating tertiary amines, such as VER, LID and DZ (*Li et al. 1995, Iamoko et al. 1990*). Competitive inhibition might be responsible for the interaction between them since two or more drugs can compete for the same enzyme.

In addition, it has been shown that tertiary amines such as LID (*Saville et al. 1989*), DZ (*Carrum et al. 1986, Hussaine et al. 1992*), diphenhydramine analog (*Bast et al. 1984 and 1990*) and tricyclic antidepressants (*Murray et al. 1992*) are capable of inactivating CYP isozymes. It is believed that mechanism-based inhibition may occur when the drug is metabolized by a CYP system to active metabolites that bind to the enzyme and cause irreversible loss of function (*Murray 1997*). Inactivation of the CYP isozymes can lead to a decrease in clearance of an administered drug, which is metabolized by the same enzymes.

VER can be co-administered with LID, DZ and DPH in clinical practice. Therapeutically, an antiarrhythmic drug can be co-administered with a calcium channel blocker for treating serious ventricular arrhythmias (*Fyke et al. 1983, Kapur et al. 1984, Mikawa et al. 1997*). Two calcium-channel blockers have been used in combination for the treatment of hypertension that offer potential advantages (*Saseen et al. 1996).* Therefore, it is quite possible that VER and LID may be given to patients with cardiac diseases. A combination of VER and DZ may be given to patients with hypertension. DPH, an over-the-counter antihistamine, is likely to be used for the treatment of nausea and vomiting associated with myocardial infarction and multi-drug therapy involving VER and DPH. Since VER has a relatively low therapeutic index (*Somogyi et al. 1981*), an understanding of the possible effect of these tertiary amines on the metabolism of

VER would help to optimize its therapy. The potential effect of LID, DZ and DPH on the metabolism of VER is not known and deserves more study.

1.7 Surface activity

VER has a characteristic amphipathic structure consisting of the aromatic termini on juxtaposition known as lipophilic groups and a positive tertiary amine group, known as hydrophilic group (Figure 1.1). The ionization and surface properties of VER were first investigated by Retzinger *et al.* (*1986*). They observed that this amphipathic structure would produce a cationic surfactant and the affinity toward an amphiphilic surface was pH dependent. The surface has a higher affinity and capacity for the neutral form of this molecule. The surface properties of VER may contribute to its diverse biologic activities, including membrane accumulation (*Scheufler et al. 1990*), induction of enzyme leakage from intact myocardium (*Cohen et al. 1981*), hemolysis (*Kamp et al. 1984*), inhibition of myocardial contractility (*Cohen et al. 1987*), and the alterations of platelet structure and function (*Retzinger 1992*). Even the ability to reverse multi-drug resistance, which is well accepted due to the inhibition of P-glycoprotein (*Plumb et al. 1990*), has been argued to be due to a generalized detergent-like (*Cano-Gauci et al. 1987*) or a specific surfactant interaction with lipids (*Seydel et al. 1994*). Later Hasegawa *et al.* (*1984*) and Surakitbanharn *et al.* (*1995*) also observed that VER might self-associate as a dimer and this self-association could affect the physical properties of the drug such as solubility.

Nonspecific adsorption will occur if a solute is surface active (*Reminton et al. 1990*). The adsorption behavior has been of long-standing interest to chemists, biologists, and engineers in chemical industries. Significant binding of VER to ultrafiltration membranes and devices was reported (*Zhirkov et al. 1984*). Adsorption of a drug onto perfusion sets is a problem, which can alter the dosage present (*Winsnes et al. 1981*). Although it has been reported that adsorption can create a problem for accurate measurements of a drug (*Cotham et al. 1975*), little attention has been paid to the influence of this crucial variable during quantitative analysis.

1.8 Rational for choosing rat as animal model

Mechanistic studies in human are extremely difficult, if not impossible, given the complex nature of the kinetics of drugs. The rat is an important animal model used in pharmacokinetic study. It has been used to study the biotransformation of VER (*McIlhenny et al. 1971, Manitpiskul et al. 1993, Mehvar et al. 1994, 1995 and 1996, Bhatti et al. 1997, Hoffman et al. 1995, Nelson et al. 1988*). A common feature shared by rats and humans is that extensive and stereoselective liver metabolism exists in both species. Nelson *et al.* (*1988*) found considerable similarities in the metabolic pathway for O-demethylation of the two species and concluded that a similar set of cytochrome P450 isozymes might be responsible for this biotransformation. A study by Hoffman *et al.* (199*5*), using absorption probes and portal blood sampling, has shown that similar to human, VER was completely (98%) absorbed in rats.

A difference in stereoselective elimination of VER has been reported between rats and humans (*Mehvar et al. 1994, Bhatti et al. 1997*). In the rat, the systemic clearance of R-VER was 48% higher than that of S-VER and the oral clearance of R-VER was over 2.5 times that of S-VER. The bioavailability of S-VER was found to be almost twice as high as that of R-VER (*Bhatti et al. 1997*). After a constant infusion of VER to isolated rat liver, perfused with a bovine albumin containing perfusate, the metabolism of racemic

VER was stereoselective in favor of R-VER (*Mehvar et al. 1994*). In addition, opposite to human, the plasma protein binding of S-VER is higher than that of R-VER. When the calculations were based on the free enantiomers, consistent with humans, the liver metabolism showed preference for S-VER (*Mehvar et al. 1994*). In accordance with these findings, a further study demonstrated that stereoselective elimination was reversed when the perfusate containing BSA was changed to a protein free perfusate. Thus, protein binding is the main determinant of stereoselectivity in the hepatic elimination of VER and therefore, in inter-species differences (*Mehvar et al. 1996*).

Time-dependent kinetics of VER have previously been demonstrated in rats using isolated perfused livers (*Mehvar et al. 1994*). However, no further mechanistic investigation has been conducted. It is unknown that if the time-dependent kinetics of VER in human can be replicated in intact rats, and if so, it is conceivable that the rat may serve as a suitable animal model for human for studying nonlinear VER kinetics.

1.8.1 Isolated perfused rat liver model

Isolated perfused rat liver simulates condition *in vivo* more than any other *in vitro* metabolic techniques. This technique permits the study of kinetics and dynamics of transport, binding and metabolism of drugs under the normal hepatic architecture, microcirculation, bile production, enzyme distribution, cell polarity and cell communication (*Pang et al. 1984, Gores et al. 1986, and Ballet et al. 1991*). A single-pass system allows one to perform a mechanistic investigation at steady state under a well-defined, controlled manner. The isolated perfused rat liver has been successfully used to study enzyme inactivation, enzyme saturation and hepatic tissue binding of LD and DZ in our laboratory (*Hussian et al. 1994 and Saville et al. 1989*).

The isolated perfused liver technique has some advantages over the *in vivo* set up. For example, the blood flow through the liver and bile production can be standardized, while no extrahepatic distribution is occurring; fewer interactions with endogenous substrates are present. Finally, the composition of the perfusion medium can easily be manipulated.

1.8.2 Isolated rat hepatocytes

Since the technique was introduced in 1969, the freshly isolated hepatocyte suspension has become a frequently used model for the study of drug metabolism, hepatic uptake, excretion and toxicity. It has been increasingly used as an *in vitro* model for the evaluation of *in vivo* drug-drug interactions (*Vercruysse et al. 1994, Li et al. 1997, and Fischer et al. 1997*) and metabolic product inhibitions (*Tsao et al. 1989*).

The isolated hepatocyte system is a particular useful tool, as it combines both the inherent simplicity of all *in vitro* systems and the ability to produce the range of metabolites normally produced *in vivo*, at levels of cofactors that approximate to those found *in vivo* (*Fry et al. 1977*). This model system has provided very accurate estimates of the *in vivo* clearances for a variety of compounds (*Zomorodi et al. 1995, Hayes et al. 1995*). Most studies demonstrated that hepatocytes, in contrast to microsomes, are consistently successful in predicting *in vivo* clearances and testify to the value of using simple scaling factors based on total hepatic cellularity (*Zomorodi et al. 1995, Ashforth et al. 1995, Hayes et al. 1995, and Vickers et al. 1993*). Hepatocytes also have advantages over the isolated perfused rat liver system. Firstly, the hepatocyte-system permits an evaluation of hepatocytes as pure isolates in a controlled environment; secondly, it permits simultaneous or sequential studies of several reactions from the same batch of cells (*Berry et al. 1992*).

1.9 Hypotheses

1. VER is a surfactant. The surface properties of VER are pH dependent.

2. The degree of adsorption to container surfaces is pH dependent.

3. In rats, VER shows a time-dependent reduction in clearance after multiple oral dosing. If this hypothesis were demonstrated, then the mechanisms of the reduction would be due to:
    a. Saturable liver metabolism and extensive, but saturable tissue binding.
    b. Enzyme inactivation.
    c. Product inhibition.

4. LID, DZ and DPH lower the rate of VER metabolism through both competitive and mechanism-based enzyme inhibitions.

1.10 Objectives

1. To investigate pH effects on VER stability and surface activity.
2. To delineate the pharmacokinetics of VER enantiomers after single and multiple oral administrations to rats.
3. To explore potential mechanisms of time-dependent pharmacokinetics of VER using isolated perfused rat liver.
4. To evaluate potential effects of LID, DZ, DPH and NOR on the metabolism of VER using isolated rat hepatocytes.

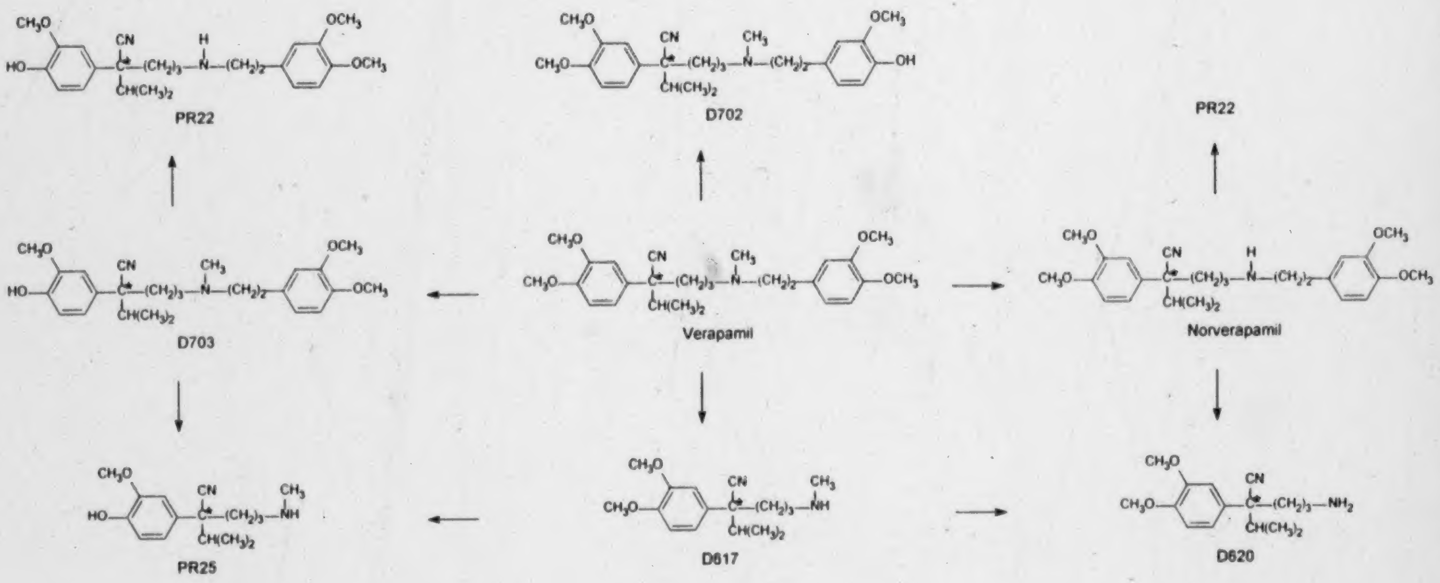

PR22
D702
PR22
D703
Verapamil
Norverapamil
PR25
D617
D620

Figure 1.1 Major metabolic pathways of verapamil in humans. (* indicates chiral centre).

2. EXPERIMENTAL SECTION

2.1 Chemical reagents

VER (racemic, R- and S- isomer) and the metabolites norverapamil [2,8-bis-(3,4-dimethoxyphenyl)-2-isopropyl-6-azaoctanitrile], D-617 [2-(3,4-dimethoxyphenyl)-5-methylamino-2-isopropylvaleronitrile], and D-620 [5-amino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile] as hydrochloride salts were kindly supplied by Knoll AG (Ludwigshafen, Germany). [^{3}H]VER (N-methyl-[^{3}H]VER; specific activity 81.1 Ci/mole, purity 97%) was purchased from Dupont (Boston, MA). Internal standard, (+)-glaucine, was purchased from Sigma (St. Louis, MO, USA). Solvents such as hexane, isopropanol (EM Science (Gibbstown, NJ, USA), acetonitrile (Fisher Scientific, Nepean, Ontario, Canada) were HPLC grade. Triethylamine and all other reagents such as orthophosphoric acid, potassium dihydrogen phosphate and potassium bicarbonate (all supplied by BDH Inc. Toronto, Canada) were of analytical grade and available commercially.

DPH was supplied by Parke Davis (Ann Arbor, MI, USA). DZ was purchased from Sigma (St. Louis, MO, USA). LID was a gift from Astra Pharmaceuticals (Mississauga, Ontario, Canada). Bio-Rad reagent was supplied by Bio-Rad Laboratories (Richmond, CA, USA), sodium heparin solution by LEO Laboratories Canada Ltd. (Ajax, Ontario, Canada) and saline by Baxter Co. (Toronto, Ontario, Canada). Hepatocyte medium, collagenase enzyme (type IV), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA).

2.2 Stability studies of verapamil

2.2.1 Sample preparation

All stock samples were prepared in polypropylene tubes (Sarstedt Inc., St. Laurent, Quebec, Canada). D-517, an analogue of VER was chosen to be the internal standard. Stock solutions of VER were prepared in 0.06 M citric-phosphate buffers with pH ranging from 2.2 to 8 to yield a final concentration of 0.5 μg/ml (base equivalent). The ionic strength of the buffer was adjusted to 0.05 M/L with NaCl. Aliquots of individual stock solutions were filtered through a cellulose acetate membrane (25mm, 0.20 Micron, Corning Glass Works, Corning, NY, USA). Other stock solutions were prepared in the same way, except 1.75% Tween 20 or 2% BSA (V/V) were included. VER solutions with different pH values were analyzed in the following manner:

One ml of each stock solution was pipetted to a new glass tube (16 × 100 mm, Kimax®, Kimble, IL, USA). After adding 50 μL D-517 (2.5 μg/ml), all samples were alkalinized to pH 13 by adding 1ml 1N NaOH. After vortexing for 1 min (some samples were kept at 4 °C for 15 hours before extraction), 3 ml methyl-butyl ether was added to the samples. After vortexing for 5 min and centrifugation for 10 min at 2,500 g, the aqueous layer was frozen in a dry ice-acetone bath. The organic layer was then decanted into a clean glass tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 200 μL of mobile phase and 100 μl was injected into the HPLC.

2.2.2 Air/aqueous interfacial tension measurement

Air to aqueous interfacial tension measurements were performed using the axi-symmetric drop shape analysis-pendant drop technique (*Rotenberg et al. 1983, Cheng et al. 1990*). Drops of sample were formed from a clean, 26 gauge Teflon cannula immersed in water in a 20ml quartz glass cuvette at ambient temperature (24 ± 1°C). Drop profile images were collected and digitized over 15 min and shape analysis was carried out using a Sun Sparc 10 work station which generated a spline curve that fitted the drop profile coordinates. The accuracy of the technique is of the order of ± 0.1 mN/m (*Li et al. 1995*). The average of at least six measurements of each drop was determined. Equilibrium at the interface was obtained after 10 min as determined by kinetics of interfacial tension observation as a function of time. Samples with different concentration of VER in water and samples with same concentration (0.5 μg/ml or 50 μg/ml) of VER in different pH citric-phosphate buffer were tested.

2.2.3 Pipette tip adsorption test

To test the potential adsorption of VER to the pipette tip (Costar, Universal Fit Pipette Tips. Corning Costa Co., Cambridge, MA, USA. 1000 μL capacity, polypropylene), which was used to transfer aqueous VER solution in all studies, parallel radioisotope measurements were performed with samples derived from the same stock with or without transferring using a pipette. The stock samples were prepared directly in the scintillation vials including 1ml VER (0.5 μg/ml) solution at different pH values with or without 2% BSA and 50 μL trace ^{3}H-VER (N-methyl-[^{3}H]VER; specific activity 81.1 Ci/mole). After 1min vortexing, half of each sample (0.5 ml) was quickly pipetted into a new scintillation vial. The contact time of each sample in the pipette tip was less than 2 seconds. 15 ml EcoLite+^{TM} liquid scintillation cocktail (ICN, Costa Mesa, CA, USA) was added to both original and transferred samples. ^{3}H radioactivity was counted using a Wallac Model 1410 liquid scintillation counter (Wallac Oy, Turku, Finland). The stability of VER in pipette tips with or without 2% BSA was studied at room temperature. Serial samples were taken immediately before and at predetermined times from pipette tips, which were loaded with 1ml of 500 ng/ml VER and trace amount of ^{3}H-VER in distilled water. ^{3}H radioactivity was counted in a similar manner. Counts for the labelled solutions were in the range of 5534 – 12150 dpm from a 1 mL sample.

2.3 *In vivo* studies in rats

2.3.1 Animals

Male Sprague-Dawley rats (250-290 g) were obtained from the Biosciences Animal Services, University of Alberta and housed in individual shoebox style cages on sterilised aspen chip bedding. They were fed with a Richmond Standard 4.5% rodent diet (PMI Nutrition International Inc., St. Louis, MO, USA) and regular tap water *ad libitum*, but food was withdrawn 12 hr before the pharmacokinetic experiment. Prior to and during the surgery, the rats were anesthetized with methoxyflurane (Janssen Pharmaceutica, North York, Ontario, Canada). Each rat was catheterized with a silastic tubing (0.025" id. x 0.047" od., Dow Corning Co., Midland, MI, USA) at the right jugular vein, after which the animal was allowed to recover for 48 hours. Physiological parameters such as body weight, white blood cell count, red blood cell count, hemoglobin content and hematocrit were monitored (Coulter Counter M430, Coulter Electronics Inc., FL, USA) the day before experiment in all rats. All the kinetic studies were carried out in the morning after an overnight fast and animals were given water *ad libitum* throughout the experiment, but food was withheld.

2.3.2 Dose preparation

VER oral solutions (10 mg/ml) were prepared by dissolving the drug in sterilized water. VER solutions for intravenous administrations (1.0 and 2.5 mg/ml) were prepared by dissolving the drug in normal saline and subsequently filtering through a membrane filter (Millex-GS, 0.22 μm, Millipore Corp., Bedford, MA, USA).

2.3.3 Experimental protocol

Rats were randomly assigned to receive one of the following two treatments: water (Control, n = 10) and five oral doses of 10 mg/kg racemic VER solution (Test, n = 10) once every 8 hours. After the pre-treatment, both groups received 10 mg/kg racemic VER in solution by gavage using a steel ball-tipped feeding needle. This dosage regimen was chosen to achieve plasma levels that are within therapeutic levels in humans. Blood samples (0.3 ml) were collected from the jugular vein just prior to dosing and at 10, 20, 30, 60, 120, 180, 240, 360 and 480 minutes. The blood samples were centrifuged at 13,000 g and the plasma portion was separated and immediately frozen at -20°C until analysis.

After the oral study the rats were allowed a 7-day washout period. The rats that continue to have working catheters were used for an intravenous dose study. The rats received one dose of either 1.0 mg/kg (n = 3) or 2.5 mg/kg (n = 3) racemic VER solution in saline through the jugular vein catheter. Blood samples (0.3 ml) were collected from the jugular vein just prior to dosing and at 2, 10, 20, 30, 60, 120, 180, 240, 360 and 420 minutes.

All kinetic studies were carried out in the morning after an overnight fast and the animals were given water *ad libitum* throughout the experiment, but food was withheld for 8 hours prior to the study.

2.3.4 Plasma protein binding

The free fractions of VER enantiomers in rat plasma were determined by an equilibrium dialysis method using the Spectrum (Los Angeles, CA, USA) equilibrium dialysis apparatus equipped with Sigma Diagnostics dialysis membranes (St. Louis, MO, USA). A molecular weight cutoff of 6-8 kD was used. Blank blood samples were collected from 6 rats through the inferior vena cava and plasma samples were obtained after centrifugation at 13,000 g. Pooled rat plasma was spiked with racemic VER at enantiomeric concentrations of 125, 250, 500, and 2000 ng/ml. The samples were then dialyzed against isotonic Sörensen buffer (pH 7.4) for 6 h at 37 °C. The dialysis cells were rotated at a speed of 15 r.p.m. After dialysis, samples from both sides of the cells were collected for HPLC analysis of VER enantiomers. Adsorption to the dialysis device and the membrane was negligible (<1%). No appreciable volume shift was found in these studies (<5%).

2.3.5 Sample preparation

All samples were processed in glass tubes (16 × 100 mm, Kimax®, Kimble, IL, USA). To 200 μl of plasma-sample, 50.0 μl of 800 ng/ml (+)-glaucine internal standard solution, 200 μl of sodium phosphate buffer (pH 7.0, ionic strength 0.1), 20 μl of 2N

NaOH and 2 ml of hexane were added. After vortexing for 5 min and centrifugation for 10 min at 2,500 × g, the aqueous layer was frozen in a dry ice-acetone bath. The hexane layer was then decanted into a clean glass tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 200 μl of mobile phase and 150 μl was injected into the HPLC system.

2.4 Liver perfusion studies

2.4.1 Animals

Male Sprague-Dawley rats (Biosciences Animal Services, University of Alberta, Canada), weighing 200-220 g, maintained on rat chow (Wayne Rodent Plox 8604-00, Continental Grain Company, Chicago, IL, USA) were used in this study. The animals were housed in the Dentistry-Pharmacy animal facility for at least 3 days before an experiment. Food and water were supplied *ad libitum*.

2.4.2 Adsorption test

Adsorption of VER to the perfusion apparatus was tested at 37 °C for 120 min. Preliminary study showed that approximately 25-27% of VER were adsorbed to the perfusion tubing (0.812" id × 0.149" od, length 387 cm, Tygon®, Cole Parmer Instrument Co., Chicago, IL, USA) during 120 min perfusion at concentration range from 2 μg/ml to 20 μg/ml, when they simply passed through the apparatus itself (Figure 2.1). This problem was circumvented by taking inlet samples (C_{in}) from a side port close to the portal vein. The concentration of VER in the perfusate leaving the liver was measured by sampling the hepatic effluent through a Teflon® tubing (Waters Corporation, MA, USA;

length: 387 cm) which showed no significant adsorption during the adsorption test. Since a fast equilibrium (~3 min) of VER adsorption onto Tygon tubing was reached in the concentrations tested (Figure 2.1), this time delay was ignored in the calculation of time to reach steady state for a perfused liver (T_{ss}).

2.4.3 Isolated rat liver perfusion studies

A "one-pass" liver perfusion method was used in this study. Details of the isolated liver perfusion procedure were provided previously (*Tam et al. 1987*). Concisely, the portal vein was cannulated with an intravascular "over-the-needle" 16 G Teflon catheter (Quick-cath, Baxter Healthcare Co., Deerfield, IL, USA) which was used to provide oxygenated hemoglobin-free Kreb's bicarbonate buffer delivered at a constant flow of 30 ml/min. The effluent perfusate sample was collected from a catheter located in the thoracic inferior vena cava. The viability of the liver was evaluated by monitoring oxygen consumption, intra-hepatic pressure, levels of aspartate (AST), and alanine (ALT) amino transferases in the effluent perfusate at zero and 120 minutes, and the general physical appearance of the organ. The rate of oxygen consumption was in the range of 2.95 ± 0.25 ml oxygen/hr/g liver (*Bloxam 1973*). AST, ALT levels and hydrostatic pressure were constant throughout the experiment. Furthermore, the stable concentrations of VER and its metabolites that was observed at steady state during the infusion period served as an indicator of liver viability.

Livers obtained from rats were randomly assigned to one of the eight groups (n= 3 to 4 in each group). Experimental groups differed from each other in the concentration of VER (2.2-43.3 μM) that was infused. VER solution was infused *via* the portal vein at a constant flow rate of 30 ml/min *via* a Vario pump (Cole Parmer Instrument Co., Chicago, IL, USA) for 2 hours. This time period was found to be sufficient for VER and its metabolites to approach a steady state. Inlet samples were taken at 0, 2, 10, 20, 40, 60, 80 and 120 minutes after the initiation of VER infusion. The concentration of VER in the perfusate leaving the liver was measured by sampling the hepatic effluent at 0, 1, 3, 5, 7, 10 minutes and at 5 minute intervals up to 60 minutes and then at a 10 minute interval up to 120 minutes. After each experiment the liver was blotted dry and its weight was recorded.

2.4.4 Stop infusion study

This experiment was designed to evaluate the binding of VER in the isolated perfused rat liver. Livers from three rats were infused with VER at 45 μM C_{in} up to 60 min until steady state was achieved. Then the drug and the metabolites were allowed to wash out by perfusing the liver with plain buffer for a period of 60-90 min at the same perfusion rate. Samples of the effluent were taken during the perfusion of drug at 0, 1, 3, 5, 7, 10 minute and at a 5-minute interval up to 60 minutes. During the washout periods, effluent samples were collected at 10 minutes intervals up to 60 minutes. Inlet samples were collected at 0, 2, 10, 20, 40, 60 minutes during drug infusion to monitor the stability of concentration of VER in the inlet.

2.4.5 Pretreatment study

To study the effect of VER on its own metabolism, rats (n = 6 per group) were randomly assigned to either a control or treatment group. VER (10 mg/kg) in saline (0.5 ml) or equal volumes of saline solution were administered to rats intra-peritoneally (i.p.)

once a day for 5 days. Liver perfusion studies were performed 12 hours after administration of the last VER or saline dose. The C_{in} of VER was 8 μM.

2.4.6 Sample preparation

For the quantification of racemic VER, NOR, D617 and D620, 100 μL of D517 (20 μg/ml) was added to a 1 ml aliquot of rat liver perfusate samples. One ml of 1 N NaOH was added to adjust pH of the sample to 12. Each sample was extracted with 3 ml methyl-butyl ether by vortexing for 5 min on a vortex shaker. After centrifugation at 2,500 × g for 10 min, the organic layer was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 200 μl of 0.01N HCl and 150μl was used for HPLC analysis.

The concentration of VER and NOR enantiomers in the liver perfusate were determined using a chiral HPLC method. To 1 ml perfusate sample, 100 μl of 500 ng/ml (+)-glaucine internal standard solution, 1ml of 1 N sodium hydroxide solution, and 3 ml of ether were added. After vortexing for 5 min and centrifugation for 10 min at 2,500 g, the aqueous layer was frozen in a dry ice-acetone bath. The ether layer was then decanted into a clean glass tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 200 μl of mobile phase and 150 μl was injected in the HPLC system.

2.5 Isolated rat hepatocyte studies

2.5.1 Hepatocyte preparation

Isolated hepatocytes were prepared from 245-300 g male Sprague-Dawley rats (Biosciences Animal Services, University of Alberta, Canada). Hepatocytes were isolated according to the method introduced by Seglen (*1976*) with slight modifications. The rat liver was perfused through the portal vein in a single-pass fashion at a flow rate of 30 ml/min for 5 min with a 37°C Ca^{2+}-free Hank's balanced salt buffer supplemented with 0.5 mM EGTA, 10 mM HEPES, 4.2 mM $NaHCO_3$, 5 mM glucose. The perfusate was changed to Hank's-collagenase buffer containing 4 mM $CaCl_2$ and 0.05% collagenase for 9 min at 10 ml/min. Hepatocytes were liberated from the perfused liver by blunt dissection and dispersed by gentle shaking in Hank's-collagenase buffer at 37°C for 1.5 min. After filtration through a gauze, the filtrate was collected. The hepatocytes were separated from non-parenchymal cells by centrifugation at 50 g for 1 min, a process that was repeated three times, each time after washing and resuspending the cells in hepatocyte medium. Gross cellular damage was measured using the trypan blue method (*Schwenk et al. 1976*). The percentage of cells that excluded trypan blue was determined in a hematocytometer under light microscopy. Viability of the hepatocytes used was greater than 90%.

2.5.2 Verapamil recovery

To estimate recovery of VER from hepatocytes, immediately after 1ml hepatocyte suspension was added into 100 µl of VER solution (final concentration: 5, 25, 50 µM) with mixing, 1 ml NaOH (1N) was added to the hepatocyte suspension to stop the reaction. Recovery was estimated by comparing to known VER standards.

2.5.3 Drug interaction studies

The incubation conditions for VER were chosen on the basis of linearity between the disappearance rate of VER with cell concentrations and incubation time. These conditions were determined in preliminary experiments. The experiment was performed under a constant flow of carbogen at 37°C in a shaking water bath (100 osc/min). The cell suspension was diluted with hepatocyte medium to a final concentration of 2.5-3 × 10^6 cells/ml. One ml of cell suspension was added to 100 μL of the hepatocyte medium containing racemic VER and one of the tertiary amines. Racemic VER concentrations were 5, 10, 25, 50 μM. At each substrate concentration, carrier solvent (PBS) or one of four concentrations 5, 10, 25 and 50 μM of each tertiary amines was included. The incubation lasted for 5 min. The reaction was terminated by the addition of 1N NaOH (1 ml) and by placing the samples in a dry ice/acetone bath. To rupture cell membranes, the suspension was frozen and thawed three times. The samples were stored at – 20 °C until assayed.

To examine the likelihood of mechanism-based inhibition, the concentration of tertiary amines was chosen at 5 times that of VER. One ml hepatocyte suspension (2.5 × 10^6 cells/ml) was preincubated with carrier solvent (PBS) or with one of the tertiary amines (final concentration: 25 μM) for 10 min at 37 °C. After 10 min, VER (final concentration: 5μM) was added to the suspension to initiate the reaction. The incubation was continued for another 5 min, and terminated by the addition of 1 ml NaOH (1N) and by placing the sample in a dry ice/acetone bath. To rupture cell membranes, the suspension was frozen and thawed three times. The samples were stored at – 20 °C until assayed.

2.5.4 Uptake study

Three ml of cell suspension (6.6×10^5 cell/ml) was plated in a 60 × 15 mm culture dishes. After a 5-h attachment period, the plating medium was removed, the attached cells were washed with prewarmed medium to remove nonadherent cells, and fresh medium was replenished. Cells formed a confluent monolayer on the bottom of the dish after 12 hours of incubation in an incubator with an atmosphere of 95% O_2 and 5% CO_2 at 37°C.

Culture dishes were removed from the incubator and washed three times with Kreb's-bicarbonate buffer at 37°C. To determine the initial velocity of VER uptake, hepatocyte cultures were incubated with 50 μL trace ^{3}H-VER and various amounts of nonlableled VER (final concentration: 0.5-50 μM). The possible competitions between VER and DZ, LID, DPH and NOR for the uptake were conducted in parallel incubations with hepatocyte culture for VER uptake. Each tertiary amine (200 μM) was individually incubated in the presence of a fixed concentration of VER (50 μM) and 50 μL ^{3}H-VER. Transport was stopped at various time intervals from 5 to 60 seconds by removing the incubation medium and rapidly washing the cells three times with 0.9% NaCl at 4°C. Preliminary study using a control dish indicated that 98% of radioactivity had been washed out by this procedure. Cells were removed from the plate with 1 ml of 1N NaOH. ^{3}H-VER radioactivity in the cells was determined using a Wallac Model 1410 liquid scintillation counter (Wallac Oy, Turku, Finland). In order to determine the stereoselectivity of VER uptake, aliquot of cell pellet was extracted and the peak areas of S- and R-VER were determined by a stereospecific HPLC analysis (section 2.6.2).

2.5.5 Determination of cell protein content

Cell protein was quantified using the Bradford method (*1976*). A standard curve was prepared using bovine serum albumin (Sigma Diagnostic, St. Louis, MO, USA). Bovine serum albumin powder was reconstituted with an appropriate volume of water to give a stock solution of 400 μg/ml. Aliquots of the stock solution (0, 2, 4, 6, 8, 10 and 12 μL) were added in triplicates to the wells of a microtitre plate (Cell Well™ 96 well plate, Corning Laboratory Sciences Co., Richmond Hill, Ontario, Canada). Sufficient volumes of the Bio-rad reagent (Bio-Rad Laboratories, Richmond, CA, USA) were added such that a final volume in each well was 200 μL. For the determination of protein concentration of cell protein, the cell pellets were lysed in 1N NaOH and diluted with H_2O by a factor 4 to 5 times. An aliquot (10 μL) of each diluted sample in triplicate was mixed with 190 μL of the Bio-Rad reagent. Absorbance readings were taken at 590 nm using Molecular Devices MAXline microtitre plate reader.

2.5.6 Sample preparation

Each sample was spiked with 50 μL of the internal standard (1500 ng/ml (+)-glaucine). Before extraction the sample was alkalinized with 500 μL of 2N NaOH. After adding 3 ml of methyl-butyl ether, the mixture was vortexed for 5 minute and then centrifuged for 10 minutes at 2,500 × g, the aqueous layer was frozen in a dry ice-acetone bath and the ether phase was then transferred into a clean tube. The organic phase was evaporated to dryness. The residue was reconstituted with 200-300 µl of mobile phase. An aliquot of these samples was analyzed using the HPLC system. The calibration curves were linear in the range of 5-1000 ng/ml for the enantiomers of VER and in the range of 10-1000 ng/ml for the enantiomers of NOR.

2.6 HPLC assay

2.6.1 Assay of racemic verapamil and its metabolites

The HPLC system equipped with a SIL 9A automatic injector (Shimadzu), a Model 501 pump, a Model 470 scanning fluorescence detector and IBM-compatible PC with a Baseline 810 data processing software (Waters, Mississauga. Canada) was used. The detector was set at λ_{ex}=230 nm and λ_{em}=320 nm and chromatographic separation was achieved on a reversed-phase LiChrospher® 60 RP-Select B column (5 µm, 125 mm × 4 mm, Merck, Darmstadt, F.R., Germany). Mobile phase was comprised of an acetonitrile-aqueous solution (26:74, v/v) (pH 3). The aqueous solution contained 0.065 M KH_2PO_4, 0.08 M H_3PO_4 and 0.0082 M Na-pentanesulphonic acid.

2.6.2 Stereospecific HPLC analysis of verapamil and norverapamil

Concentrations of S- and R-VER and S- and R-NOR were determined using a stereospecific high-performance liquid chromatographic (HPLC) method. The assay developed by Shibukawa and Wainer (*1992*) was adapted for this study. The enantiomers of VER and NOR were separated on a silica column (Supelcosil LC-Si) and a Chiralpak AD column. The mobile phase was comprised of hexane-isopropanol-ethanol (85:7.5:7.5, v/v/v) containing 1.0% triethylamine. The column temperature was kept at 30°C, and the flow-rate was maintained at 1.0 ml/min. The excitation and the emission wavelengths of the fluorescence detector were set at 272 and 317 nm, respectively, with the bandwidth of 18 nm. VER eluted faster than NOR and for both VER and NOR, the S-enantiomer eluted faster than the R-enantiomer. No significant interference of plasma components was observed. The limit of quantification of the assay was 5 ng/ml for the enantiomers of VER and 10 ng/ml for the enantiomers of NOR when a plasma volume of 200 μL was used.

2.6.3 Standard curves

Standard stock solutions of VER and its metabolites were prepared separately in 0.01N HCl to a final concentration of 0.5 mg/ml base equivalent and stored at -20°C. On the day of analysis, a working stock solution was prepared by mixing appropriate aliquots of individual stock solutions of VER and its metabolites and diluted with an appropriate drug-free matrix (citric-phosphate buffers with or without 2% BSA, canine plasma, liver perfusate or hepatocyte medium). The calibration curves were linear in the range of 5-1000 ng/ml for the enantiomers of VER ($r>0.999$), 10-1000 ng/ml for the enantiomers of NOR ($r>0.997$), and 10-500 ng/ml for D617 ($r>0.999$) and D620 ($r>0.998$). To establish the accuracy and precision of the assay, a validation study was performed using quality control (QC) samples prepared by other colleagues in the laboratory. The concentration of these QC samples covered the range of the calibration curve (10, 30, 200, 1000 ng/ml of VER and NOR enantiomers; 10, 30, 100, 200 ng/ml of D617 and D620). The intra- and inter-day coefficients of variation for accuracy and precision were < 10% for D617, D620 and VER enantiomers, and < 18% for NOR enantiomers at all the concentration analyzed.

2.7 Pharmacokinetic analysis

2.7.1 *In vivo* studies in rats

The area under the plasma concentration-time curve (AUC) and the area under the first moment curve (AUMC) of VER and NOR enantiomers were calculated using WinNonlin (*Weiner and Stucker 1996*). The non-compartmental model was used for the calculation of pharmacokinetic parameters. For the single dose regimen the oral clearance was calculated using equation 2.1:

$$CL_o = D_{oral}/AUC_{0-\infty} \qquad (2.1)$$

where D_{oral} is the enantiomeric dose administered orally. In all cases, the plasma concentration of the last time point was either very close to, or below the quantifiable limit. Determination of the slope of the terminal phase was hampered by the huge fluctuation in concentration. Thus it was assumed that $AUC_{0-tlast} = AUC_{0-\infty}$.

For the multiple dose study, steady state was assumed to occur after 5 oral doses were given every 8 hours. Since the last sampling time point was the same as the dosing-interval, the oral clearance (CL_o) of VER therefore was computed according to equation 2.2:

$$CL_o = D_{oral}/AUC_{0-last} \qquad (2.2)$$

For the intravenous dose, the elimination rate constant (k_{el}) was calculated from the slope of the terminal phase. Half-life ($t_{1/2}$), apparent systemic clearance (CL_s), mean residence time (MRT), and volume of distribution at steady-state (V_{ss}) were computed using the following equations:

$$t_{1/2} = Ln2/k_{el} \qquad (2.3)$$

$$CL_s = D_{iv} / AUC_{0-\infty} \quad (2.4)$$

$$MRT = AUMC_{0-\infty}/AUC_{0-\infty} \quad (2.5)$$

$$V_{ss} = MRT*CL_s \quad (2.6)$$

F, the absolute bioavailability of VER was calculated by matching the oral data to the i.v. data of the same rat under an assumption of linear kinetics as:

$$F = (D_{iv}/ AUC_{oral})/(D_{oral}/ AUC_{iv}) \quad (2.7)$$

2.7.2 Liver perfusion studies

The time to reach steady state ($T_{ss},$) was determined statistically using the procedure reported by Saville *et al.* (*1987*). The mean outlet concentration (C_{out}) during the steady-state period of VER and its metabolites were calculated by averaging the concentration data from the time to reach steady state to the end of infusion. The extraction ratio for VER at steady state, E was calculated as:

$$E = (C_{in}-C_{out}) / C_{in} \quad (2.8)$$

where C_{in} was the mean inlet concentration during the steady-state period. Hepatic intrinsic clearance (CL_{int}) was calculated as follows:

$$Cl_{int} = QE/F \quad (2.9)$$

where Q was the volumetric flow rate through the liver, F = 1- E.

The velocity of metabolism of VER at steady state (ν) and its concentration at the enzyme site can be described as:

$$\nu = Cl_{int}C_{ss} \quad (2.10)$$

where C_{ss} is the steady-state concentration of the enantiomer in the liver, which according to the well-stirred model is equal to the concentration leaving the liver (C_{out}) (*Pang and Rowland 1977*). Therefore, equation can be rewritten as:

$$\nu = CL_{int}C_{out} \qquad (2.11)$$

The steady-state elimination velocities of VER required a mixed elimination model:

$$\nu = CL_2\, C_{out} + V_{max} * C_{out} / (K_m + C_{out}) \qquad (2.12)$$

where CL_2 represents the clearance by a low affinity, high capacity site and, because saturation of this binding site is not evident over the concentration range studied, it represents the sum of all apparent first-order clearance values for elimination. V_{max} and K_m values are pooled Michaelis-Menten parameters at steady state. The fit of the data to this mixed model was significantly better than a single linear or a single Michaelis-Menten model, judged by lower Akaike's Information Criterion (AIC) and Schwarz Criterion (SC) values and higher correlation coefficients. Parameter estimation was performed by nonlinear least square regression of unweighted data using WinNolin (*Weiner and Stucker 1996*).

The amount of VER bound and/or accumulated in the liver tissue (A_b) was determined by summation of the amount released during the washout (AUFC) and the amount metabolized at the same period (A_m):

$$A_b = AUFC + A_m \qquad (2.13)$$

$$A_m = \int_0^{60} \left[CL_2 * C_{(t)} + \frac{V_{max} * C_{(t)}}{K_m + C_{(t)}} \right] dt \qquad (2.14)$$

where AUFC was the area under the flux vs. time curve during the washout period (*Hussain et al. 1994*). These areas and the half-life for washout of VER and NOR from the livers were calculated using the computer program LAGRAN (*Bloxam 1973*). Partition coefficient (K_{tw}) was calculated as:

$$K_{tw} = A_b * \rho / C_{ss} * W * f_t \qquad (2.15)$$

where W is the weight of the liver (g), ρ, the density of the liver (g/ml) and f_t, the fraction of the liver volume occupied by liver tissues; ρ and f_t were assumed to be 1 g/ml (*Gray et al. 1987*) and 0.85 (*Goresky 1963*), respectively.

2.7.3 Isolated hepatocyte studies

Data that were derived were subject to graphical analysis as outlined in Segel (*1975*). The pooled K_m and V_{max} were determined by linear regression analysis of Lineweaver-Burk plots. The inhibition constant (K_i) was determined mathematically and graphically using Dixon plots (*Butterworth 1972*). Discrimination between types of inhibition was accomplished by visual inspection of the double reciprocal plots of the data and by comparing the estimated K_m and V_{max} values in the presence and absence of inhibitors.

The degree of inhibition *in vivo* can be predicted by the following equation, independent of the inhibition type, except in the case of uncompetitive inhibition, assuming that the substrate concentration is much lower than the K_m value (*Davila et al. 1998*):

$$\%\ \text{Inhibition} = \left(1 - \frac{v(+I)}{v(control\ )}\right) * 100 = \left(1 - \frac{1}{1 + I_u / K_i}\right) * 100 \qquad (2.14)$$

where *v(+I)* and *v(control)* represent the initial metabolic velocity in the presence and absence of the inhibitor respectively, I_u is the unbound concentration of the inhibitor. At therapeutic concentrations, the plasma protein binding values are 70% for LID (*McNamara et al. 1980*), 80% for DZ (*Boyed et al. 1989*), 80-98% for DPH (*Glazko et al. 1974*). Thus, unbound plasma concentrations of those tertiary amines will be 30% or less than the total drug concentrations. In the calculations, the total plasma concentrations in human were used. These were highly conservative estimates for the substrate concentration available to the metabolic enzymes.

The least square linear-regression was used to calculate the values of initial uptake velocities from the respective accumulation *vs.* time curves. Based on a preliminary study, which showed that the cellular uptake of VER was linear for 60 seconds, the initial uptake velocity was calculated using linear regression on points taken at 5, 10, 15, 30 and 45 seconds.

2.7.4 Statistical analysis

2.7.4.1 Stability studies

All data were presented as mean ± SD. The means were compared to the value at pH 2.2 using a two-tailed Student's *t* test. All tests were conducted at a significance (α) level of 0.05.

2.7.4.2 *In vivo* studies in rats

Because of high inter- and intra-subject variation for VER (*Eichelbaum and Somogyi 1984*), the data were first tested for normal distribution. All parameters, subjected to any statistical evaluation were found to be normally distributed by using the F test. Therefore the comparisons between the single and multiple dose regimens were made using a one-tailed Student's *t* test. A comparison between the two IV doses was made using the two-tailed Student's *t* test. All tests were conducted at a significance (α) level of 0.05. Data are reported as mean ± SD.

2.7.4.3 Liver perfusion studies

In all isolated liver perfusion study, the unpaired two tail *t* test was used to test any differences in T_{ss}, C_{out}, E, and CL_{int} between the groups in order to evaluate the effect of the pretreatment. Statistical differences between the two enantiomers of VER were analyzed using a paired, two-tailed *t* test. The effect of C_{in} on CL_{int} was analyzed using Non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA). The level of significance was set at $p = 0.05$. Values are reported as mean ± SD.

2.7.4.4 Isolated hepatocyte studies

For isolated hepatocyte studies, significance testing of K_m, V_{max} and K_i values between two enantiomers of VER was performed using the paired Student's *t* test. Percentage data were analyzed using arcsin ($\sin^{-1}(\sqrt{Y})$ transformation prior to Student's *t* test (*Steel and Torrie 1980*). Conclusions based on *t*-test of original data and of transformed data agreed with each other. Results of analysis of original data are presented in Table 3.15 and 3.16. All tests were conducted at a 0.05 level of significance. Values are reported as mean ± SD.

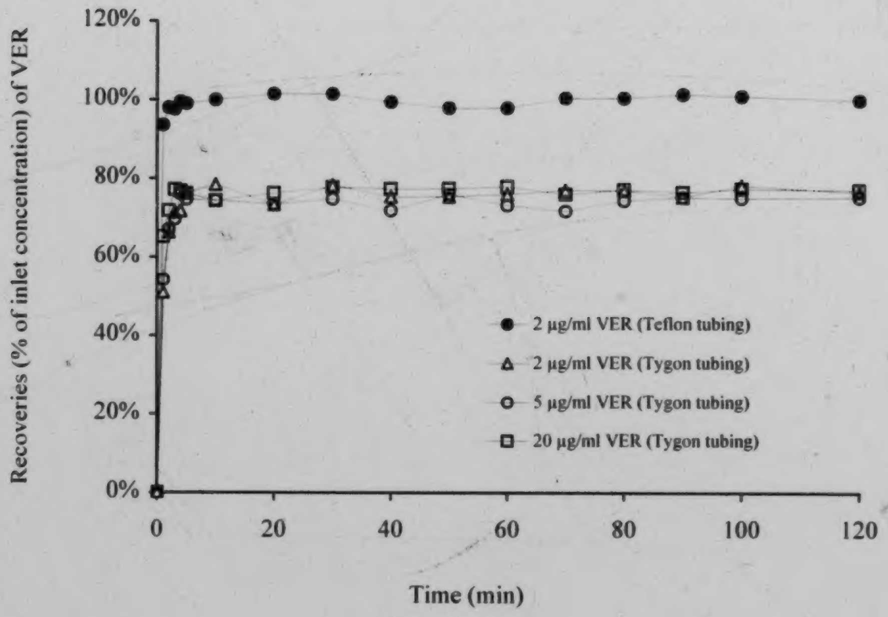


Figure 2.1 Percentage of inlet concentration after flowing VER solutions through perfusion tubings at a flow rate of 30 ml/min. The inlet concentrations were measured from the samples collected from a Kreb's buffer reservoir with 2, 5, or 20 µg/ml of VER.

3. RESULTS

3.1 Stability studies of verapamil

3.1.1 pH effect on HPLC measurements

The fluorescent intensity of VER decreased 49.7 ± 2.3% when solution pH was increased form 2.2 to 8.0. Fluorescence of D-517 (internal standard) was not pH dependent (Figure 3.1). During HPLC analysis, no extra peaks, nor change of retention time or tailing of VER peak were found for any sample at the pH range studied, indicating that no decomposition or irreversible binding to the column was involved. Table 3.1 shows the effects of filtration, surfactant and protein on the pH dependency of VER recovery after extraction. Firstly, values of VER solubility in all solutions were confirmed, as no precipitates and change in the pH dependency before and after filtration were observed. Secondly, this pH dependency was reproducible for both S-VER and R-VER with different mobile phase and different detection wavelength. Thirdly, the pH dependency did not change for samples maintained in basic solution (1N NaOH) for prolonged periods of time, excluding the possibility of slow equilibrium between the ionization states. The samples with 1.75% Tween 20 showed less pH dependency (13.8 ± 2.6 %), indicating that the pH effect was alleviated by the strong surface active agent (Figure 3.2). pH dependency was absent with samples having 2% BSA present, indicating that BSA stabilized VER in buffers (Table 3.1).

3.1.2 Surface activity

VER was surface active in the working buffer system (Figure 3.3), and the surface tension decreased by 10% when pH was increased from 3.0 to 8.0 at a concentration of 50 μg/ml. However, no significant change in surface tension as a function of pH value was found at 0.5 μg/ml of VER (The loss of fluorescent response was found at this concentration), suggesting that the fraction of the total mass of VER localized at air-water interface was very small or negligible. A radiometric method was utilized to measure the adsorption from solution at the solid/aqueous interface. The adsorption of VER to a polypropylene tube was not significant at 0.5 μg/ml, as no loss of radioactivity was noticed with increasing pH. Figure 3.4 shows the decline in concentration of VER as a function of pH and this decline in concentration occurred only for samples transferred using a pipette. Figure 3.5 shows that in the presence of 2% BSA, the concentration of VER was constant at room temperature over 25 min. In contrast, in the absence of 2% BSA, VER concentration declined within 26 min to a minimum of 64.7 ± 0.9%. There was a significant relationship between the pipette tip adsorption and the pH dependent VER depletion ($r^2 = 0.94$, $p<0.05$).

3.2 *In vivo* studies in rats

3.2.1 Oral pharmacokinetics

The plasma concentrations of VER enantiomers after single dose (10 mg/kg) and multiple dosing (10 mg/kg once very 8 hours for 5 doses) to rats are illustrated in Table 3.2, 3.3, 3.4 and 3.5. For S-VER plasma concentrations, the intra-individual coefficient of variation ranged from 42.3 to 96.1% after single dose, and 29.1 to 181.4% after multiple dosing. The intra-individual variation of R-VER ranged from 21.0 to 119.2% after single dose and 22.3 to 198.5% after multiple dosing. Figure 3.6 and 3.7 shows the mean plasma concentration-time profiles of VER and NOR enantiomers after oral administrations of racemic VER. Peak VER levels occurred at about 1 hour after the dose. The plasma levels at time 0 and at the end of the dosing interval of 8-hr were similar, indicating that apparent steady-state conditions were attained in these studies. The oral clearances of R-VER were 4 to 5 times higher than those of S-VER for both single and multiple doses, indicating that presystemic elimination of racemic VER was highly selective for R-VER. Compared to single dose, the oral clearance of S-VER after multiple dosing was significantly decreased from 253 ± 123 to 177 ± 50 ml/min/kg, while the AUC value of S-VER was increased from 72.3 ± 29.2 to 98.8 ± 29.7 μg/ml*min/kg ($p<0.05$, Table 3.6). Unlike S-VER, the oral clearance and AUC of R-VER did not change significantly between single and multiple doses, indicating stereoselective time-dependent kinetics. The AUC of NOR after multiple dosing was increased by approximately 94% for S-VER ($p < 0.05$) and 89% for R-VER ($p < 0.05$) indicating accumulation of NOR. Table 3.7 shows that the bioavailability was increased after multiple dosing. The magnitude of increase is about 54% ($p < 0.05$) for S-VER and 28% ($p > 0.05$) for R-VER. The plasma R/S-enantiomer ratio after multiple dosing showed a decrease of 26% for VER and an increase of 7% for NOR ($p > 0.05$), whereas the ratio of NOR/VER showed a significant increase after multiple dosing (Table 3.8). This indicates that the accumulation of NOR is greater than that of VER.

3.2.2 Intravenous pharmacokinetics

The mean plasma concentration-time profiles and the pharmacokinetic parameters obtained after intravenous administration of 1.0 and 2.5 mg/kg racemic VER are shown in Figure 3.8 and Table 3.9, respectively. On average, the clearance of the R-enantiomer was higher ($p<0.05$) than that of the S-enantiomer. Also, the volume of distribution of R-enantiomer was higher ($p<0.05$) than that of the S-enantiomer. The half-life of the R-enantiomer was higher ($p>0.05$) than that of the S-enantiomer (Table 3.9). The clearance values of VER after intravenous dosing tended to increase with increasing dose. For R-VER there was a significant increase in clearance between the 1.0 and the 2.5 mg/kg doses. The volume of distribution seems to be constant or slightly increased with increasing dose. The half-life ($t_{½}$) did not change significantly between the dosing regimens. After intravenous administration, NOR was too low for quantification.

3.2.3 Plasma protein binding

In rat plasma, the free fraction of S-VER was lower than that of R-VER (Table 3.10). The free fraction for both enantiomers was concentration independent in the range studied (125 ng/ml to 1000 ng/ml VER). However, there was a significant increase in the free fraction for both enantiomers when the concentration reached 2000 ng/ml.

3.3 Liver perfusion studies

Shown in Figure 3.9 is the concentration-time courses of racemic VER and its metabolites in the outlet samples from a representative isolated perfused rat liver during a constant infusion of 8.34 μM of VER for 120 minutes. Upon continuous infusion, there was a gradual reduction of extraction of VER with time and the steady-state condition was established for both VER and its metabolites approximately in 80 min. The time course of NOR and D-617 exhibited a similar trend. Concentration of D-620, a minor metabolite, increased to a maximum concentration at about 10 to 30 min, then declined to a steady state in 88% of the livers studied (28 out of 32 rats). This behavior did not follow the time course of VER. The relative contributions of NOR, D-617, and D-620 to mass balance were 11.1 ± 0.6%, 6.3 ± 1.0%, and 2.6 ± 0.9%, respectively. Unchanged VER accounted for 24.0 ± 5.7% of the infused VER at steady state.

3.3.1 Concentration dependent hepatic extraction and stereoselectivity

Figure 3.10 is the time course of the concentrations of VER and NOR enantiomers in the outlet in a representative liver during an infusion of 2.20 μM VER. Within the first several minutes of infusion of this low input concentration, only insignificant amount (below the detection limit of 5 ng/ml) of VER enantiomer appeared in the effluent. It was obvious that the outlet concentration of R-enantiomer of VER was higher than its antipode and the opposite was true for NOR. These results indicated that the metabolism of racemic VER was stereoselective in favor of S-VER at this infusion concentration. When VER steady-state outlet concentration (C_{out}) was increased from 0.21 to 20.6 μM, E declined from 0.91 to 0.41 (Figure 3.11). Values of steady-state R/S ratio decreased from 1.35 to 0.49 as C_{out} values was increased from 0.21 to 0.75 μM and these values increased and approached 1 when C_{out} values were higher than 2 μM (Figure 3.12). The results indicate that there was a stereoselective hepatic extraction and the direction of stereoselectivity changed from favoring S-VER metabolism to R-VER and stereoselectivity disappeared as C_{out} increased. Similar changes are shown in Table 3.11, in which the calculated intrinsic clearance (CL_{int}) values of S-VER decrease progressively with increasing C_{in} values. This change levels off when C_{in} exceed 12.5 ± 0.15 μM. Different from S-VER, significant decrease in CL_{int} of R-VER could only be detected among the groups with a C_{in} value ranging from 3.60 ± 0.05 μM to 16.3 ± 0.44 μM. The different changing pattern of VER enantiomers was reflected by a corresponding change in the S/R ratio of CL_{int} (Table 3.11).

Steady-state levels of NOR and D-617 formed by N-dealkylation were nonlinearly related to VER outlet concentration (Figure 3.13 and Figure 3.14). The value of material balance (MB) changed with steady-state C_{out} (Figure 3.15). At the lowest C_{in}, ~74% of the drug was unaccounted for, suggesting the presence of other unidentified pathways of metabolism. Most of these pathways were probably saturable because mass balance (MB) reached ~80% at high C_{in}.

The steady-state elimination rate of VER enantiomers exhibited biphasic kinetics. Equation 2.12 describes the data appropriately ($r^2 = 0.94$), suggesting there are linear, low affinity high capacity (CL_2) and nonlinear, a high affinity low capacity (V_{max}, K_m) pathways (Figure 3.16). The estimates of pooled steady-state parameters are K_m (S-VER: 0.082 μM; R-VER: 0.156 μM), V_{max} (S-VER: 2300 ng/min/g liver; R-VER: 3740 ng/min/g liver), and CL_2 (1.80 ml/min/g liver and 1.50 ml/min/g liver for S-VER and R-VER, respectively).

3.3.2 Effect of pretreatment

The effect of VER pretreatment on its own kinetics in liver perfusion experiments is presented in table 3.12. VER pretreatment for 5 days caused no significant changes in the kinetic parameters, except in the steady-state D-620 levels. The characteristic rise of

D-620 to a maximum before decreasing to a steady state was not observed in pretreated rats. The change in D-620 profile after the pretreatment with VER suggests enzyme inactivation for the formation of D-620. Since, this metabolic pathway accounted for a minor portion (~3.87% of C_{in} at steady state) of total VER turnover in rats, enzyme inactivation, if present, was not significant.

3.3.3 Tissue accumulation by the liver

The T_{ss} decreased and tended to reach a steady value of about 20 minutes as the C_{out} of VER increased (Figure 3.17). In the stop-infusion experiment, both R-, S-VER and NOR levels declined in a biphasic fashion after reaching a steady state, a rapid drop was followed by a slow decline over 60 min (Figure 3.18). At C_{in} 43.3 ± 3.0 μM, the total amounts of VER bound (reversible and irreversible) and /or distributed in liver tissues, as calculated from the washout curve were 594 ± 26.9 and 652 ± 26.1 nmol/g of liver for S-VER and R-VER respectively. K_{tw} values were 70.9 ± 3.0 and 73.9 ± 4.0 for S-VER and R-VER. The half-life values for washout from the liver was 15.0 ± 1.1 min and 15.6 ± 2.3 min for S-VER and R-VER respectively, and VER was observed at the outlet 90 min after infusion ceased, suggesting that VER and /or its metabolites were tightly bound to liver tissues. No significant stereoselective hepatic tissue binding and the release of bound VER and NOR from liver were found at this C_{in} (Table 3.13).

3.4 Effect of several tertiary amines on the metabolism of verapamil

3.4.1 Hepatocyte system

The recovery of VER from hepatocyte suspension was 95.0 ± 2.9%. When VER was incubated with hepatocyte medium without hepatocytes, no disappearance was seen after 1 hour. Cells that were stained with trypan blue after treatment with NaOH did not show drug metabolizing activity during 1 hour of incubation, thus permitting the use of the trypan blue exclusion test to estimate cellular drug metabolizing competence. The major metabolite of VER was identified as NOR. Other metabolites were not detected using the present analytical method.

3.4.2 Effect of co-incubation

All four tertiary amines inhibited the metabolism of S-VER and R-VER (Figures 3.19 -3.26). Lineweaver-Burke analysis reveals that the average K_m and V_{max} values for S- and R-VER are 7.37 μM and 7.85 μM, and 595 pmol/min/10^6 cells and 567 pmol/min/10^6 cells (values are the average apparent K_m and V_{max} determined in the four experiments listed in table 3.14). LID and NOR are competitive inhibitors, whereas DZ and DPH are mixed inhibitors of VER enantiomers (Figures 3.19a-3.26a). K_i-values obtained from the Dixon plots show that NOR is the most potent inhibitor of S-VER metabolism followed by, in decreasing order, DZ, DPH and LID (Figures 3.19b-3.26b). For R-VER, the most potent inhibitor is DZ followed by NOR, DPH and LID. The K_i values between S-VER and R-VER were not significantly different for the inhibition by LID, DZ, and DPH. NOR showed a lower K_i for S-VER than that of R-VER, indicating that the inhibition by NOR was favoring S-VER (Table 3.14). NOR formation rate decreased with an increasing concentration of DZ ($r^2 = 0.937$, $p < 0.05$). DPH caused an increase in NOR formation rate, but no correlation between the formation rate change and VER disappearance inhibition effect could be detected (Fig 3.27). LID had no significant effect on the formation rate of NOR. A calculation of the predicted *in vivo* inhibition of VER metabolism by the tertiary amines indicated that DZ, DPH, and NOR would inhibit VER metabolism by less than 5%, whereas inhibition by LID was less than 15% (Table 3.14).

3.4.3 Effect of preincubation

Preincubation of hepatocytes with 25 μM each of LID, DZ, DPH, and NOR prior to the addition of VER increased the degree of inhibition on the disappearance of VER enantiomers. Compared to control (0 minute preincubation), the extent of inhibition by LID, DZ and DPH was significantly increased with preincubation (Table 3.15). No significant effect was found for prolonged exposure of NOR compared to control. These results indicate that the inhibition by LID, DZ, and DPH is time-dependent, suggesting a consequence of mechanism-based inactivation of the enzymes by these tertiary amines. The degree of inhibition of NOR enantiomer formation rate increased with a longer preincubation time for LID and DZ, whereas no time effect on the formation of NOR enantiomer could be detected for DPH (Table 3.16).

3.4.4 Effect of uptake

The time courses for hepatocyte uptake of VER are depicted in representative experiments (Figure 3.28). Accumulation of VER remained linear with time up to 60 sec among all concentration examined. Values of the slope were used to estimate the initial uptake kinetics. The initial rate of VER uptake was directly proportional to VER concentration from 0.5 to 50 μM with no evidence of saturation (Figure 3.29). The uptake constant was 0.260 ± 0.033 nmoles/min/10^6 cells/μM for racemic VER. By analyzing cell pellets using HPLC, the peak ratio of S-VER *vs*. R-VER was close to unity within VER concentration studied (S-VER/R-VER: 1.05 ± 0.02 and 1.02 ± 0.01 at VER concentration 0.5 and 50 μM respectively), indicating the uptake was not stereoselective. The interference of adding LID, DZ, DPH and NOR on the uptake of VER was minimal (Figure 3.30). The results indicate that a passive diffusion process dominated the uptake process within the concentration range studied. The change in VER disappearance rate was mainly due to a difference in the metabolic rate.

Table 3.1 **Recovery of VER from different buffered solutions under different experimental conditions (n = 4).**

PH	VER concentration (ng/ml)					
	S-VER	R-VER	Filter[b,f]	Tween 20[c,f]	Extension of equilibration[d,f]	2% BSA[e,f]
2	234 ± 3.1	239 ± 1.5	489 ± 7.8	495 ± 3.5	486 ± 4.4	501 ± 5.3
3	226 ± 2.7	227 ± 3.2	459 ± 8.9	492 ± 5.3	478 ± 13.8	506 ± 4.7
4	199 ± 3.1[a]	202 ± 3.8[a]	436 ± 10.8[a]	489 ± 8.6	432 ± 11.5[a]	496 ± 5.7
5	209 ± 2.9[a]	213 ± 4.6[a]	385 ± 6.7[a]	488 ± 9.7	391 ± 10.2[a]	502 ± 3.5
6	169 ± 2.9[a]	173 ± 1.4[a]	358 ± 5.6[a]	475 ± 6.6[a]	361 ± 13.1[a]	496 ± 7.6
7	167 ± 3.5[a]	171 ± 1.6[a]	333 ± 7.3[a]	408 ± 10.2[a]	344 ± 4.2[a]	498 ± 3.9
8	152 ± 2.5[a]	156 ± 0.7[a]	320 ± 8.7[a]	398 ± 8.4[a]	322 ± 5.3[a]	503 ± 5.8

[a] Data are significant different from the value at pH 2.2 ($p < 0.05$).
[b] Samples were filtered through cellulose acetate membrane filters (25 mm, 0.20Micron, Corning, N.Y.).
[c] 1.75% Tween was added to sample stock solutions.
[d] Samples were in pH 13 for 15 hours before extraction with ether.
[e] 2% BSA was added to sample stock solutions.
[f] Values are racemic VER.

Table 3.2 Plasma concentration of S-VER *vs.* time data after a single 10 mg/kg oral dose to rats.

Rat	Plasma concentration of S-VER (ng/ml)									
	0 min	10 min	20 min	30 min	60 min	120 min	180 min	300 min	360 min	480 min
1	0.0	238.9	213.4	122.7	101.4	82.2	65.8	29.1	bql	bql
2	0.0	13.5	156.8	240.9	128.7	115.8	59.3	58.9	bql	bql
3	0.0	56.0	md	27.9	44.1	54.8	56.8	98.8	67.3	21.3
4	0.0	45.1	54.5	68.5	46.6	27.3	10.7	8.6	6.5	bql
5	0.0	42.2	76.3	65.1	65.9	53.9	41.4	30.5	bql	bql
6	0.0	102.7	141.1	45.4	81.6	29.3	38.3	14.9	13.3	11.6
7	0.0	80.6	81.1	150.1	108.5	103.6	125.0	86.0	42.0	35.9
8	0.0	46.3	97.6	100.0	149.0	42.2	91.1	22.2	25.2	bql
9	0.0	51.8	58.0	57.6	58.9	50.0	24.2	22.2	15.7	bql
10	0.0	72.8	47.0	80.6	61.9	75.4	59.6	32.8	bql	bql
Mean	0.0	75.0	102.9	95.9	84.7	63.5	57.2	40.4	24.3	22.9
%CV	0.0	83.3	54.6	65.3	42.3	47.3	57.3	75.8	96.1	53.2

bql: value is below quantifiable limit of 5 ng/ml; md: missing sample; %CV: coefficient of variation.

Table 3.3 Plasma concentration of R-VER *vs.* time data after a single 10 mg/kg oral dose to rats.

Rat	Plasma concentration of R-VER (ng/ml)									
	0 min	10 min	20 min	30 min	60 min	120 min	180 min	300 min	360 min	480 min
1	0.0	118.3	131.6	53.9	41.7	21.8	12.5	6.1	bql	bql
2	0.0	5.5	50.2	58.2	26.2	24.0	15.0	25.9	bql	bql
3	0.0	42.4	bql	bql	6.9	6.1	7.7	22.8	6.6	bql
4	0.0	18.5	22.5	22.6	11.3	8.3	bql	bql	bql	5.5
5	0.0	7.6	16.7	10.1	9.5	8.5	6.7	bql	bql	bql
6	0.0	45.5	62.3	11.9	20.0	9.1	23.6	bql	bql	6.8
7	0.0	22.0	24.1	40.5	26.0	19.9	31.1	19.9	8.9	10.7
8	0.0	13.1	27.1	25.5	33.7	9.7	48.1	7.2	bql	bql
9	0.0	5.0	7.7	6.6	8.8	bql	bql	bql	bql	bql
10	0.0	11.6	4.8	15.1	10.3	17.6	6.0	6.0	bql	bql
Mean	0.0	29.0	38.6	24.9	19.4	13.9	18.8	14.6	7.7	7.7
%CV	0.0	119.2	102.6	78.5	61.9	49.4	78.2	63.0	21.0	35.1

bql: value is below quantifiable limit of 5 ng/ml; %CV: coefficient of variation.

Table 3.4 **Plasma concentration of S-VER *vs.* time data after 10 mg/kg oral doses given every 8 hours for five doses to rats.**

Rat	Plasma concentration of S-VER (ng/ml)									
	0 min	10 min	20 min	30 min	60 min	120 min	180 min	300 min	360 min	480 min
1	7.8	82.7	92.4	40.8	74.0	64.8	63.4	46.0	bql	bql
2	0.0	75.5	191.8	228.6	276.4	120.3	152.1	93.8	bql	bql
3	0.0	76.7	72.9	70.2	59.2	73.3	56.8	66.9	9.8	bql
4	0.0	34.0	119.2	121.6	137.3	124.4	114.1	79.7	16.7	bql
5	0.0	27.0	94.6	75.7	91.3	88.8	73.0	54.0	14.2	bql
6	0.0	75.5	82.9	73.3	73.4	87.3	104.2	36.3	11.9	15.9
7	14.9	77.8	99.4	91.0	111.0	96.7	96.9	21.1	30.8	bql
8	24.5	185.4	218.3	225.7	162.1	144.7	69.1	115.9	25.8	33.0
9	0.0	47.9	94.5	127.6	221.2	157.8	51.4	17.3	bql	bql
10	0.0	135.0	187.3	199.6	116.7	91.9	48.8	31.2	bql	35.0
Mean	4.7	81.8	125.3	125.4	132.3	105.0	83.0	56.2	18.2	28.0
%CV	181.4	57.8	42.2	55.0	53.0	29.1	40.0	57.8	45.7	37.5

bql: value is below quantifiable limit of 5 ng/ml; %CV: coefficient of variation.

Table 3.5 Plasma concentration of R-VER *vs.* time data after 10 mg/kg oral doses given every 8 hours for five doses to rats.

	Plasma concentration of R-VER (ng/ml)									
	0 min	10 min	20 min	30 min	60 min	120 min	180 min	300 min	360 min	480 min
1	0.0	35.5	57.0	11.0	17.5	13.8	10.3	7.2	bql	bql
2	0.0	21.4	51.0	63.3	60.4	22.7	29.6	23.5	bql	bql
3	0.0	19.2	13.2	13.7	7.6	7.4	10.1	42.3	bql	bql
4	7.1	12.0	39.9	28.8	32.4	21.0	21.2	19.0	8.5	bql
5	0.0	13.0	31.2	23.0	23.1	22.2	16.2	12.2	bql	bql
6	0.0	19.1	20.8	15.4	12.0	19.7	54.5	7.8	5.6	10.5
7	7.5	23.4	20.0	19.6	22.2	13.5	31.4	5.4	18.4	bql
8	0.0	52.1	52.0	52.4	30.5	35.3	20.5	27.9	7.3	14.5
9	0.0	10.2	22.7	26.1	41.8	22.3	8.3	bql	bql	bql
10	0.0	27.8	33.3	37.7	15.2	11.4	bql	bql	bql	bql
Mean	1.6	23.4	34.1	29.1	26.3	18.9	22.5	18.2	9.9	12.5
%CV	198.5	54.2	44.9	59.3	60.1	41.5	65.0	70.0	57.9	22.3

bql: value is below quantifiable limit of 5 ng/ml; %CV: coefficient of variation.

Table 3.6 Pharmacokinetic parameters of VER enantiomers after a 10 mg/kg oral dose to rats. Data are presented as Mean ± SD (n =10 per treatment).

	Single Dose		Multiple Dose	
	S-VER	R-VER	S-VER	R-VER
Dose (mg)	1.40 ± 0.16	1.40 ± 0.16	1.35 ± 0.16	1.35 ± 0.16
t_{max} (min)	52 ± 72	51 ± 58	53 ± 49	81 ± 85
C_{max} (ng ml^{-1})	138 ± 66[b]	49 ± 35	154 ± 70[b]	45 ± 11
AUC (μg min $ml^{-1}kg^{-1}$)	72.3 ± 29.2[b]	19.2 ± 7.43	98.8 ± 29.7[a,b]	21.08 ± 8.72
CL_o (ml min^{-1} kg^{-1})	253 ± 123[b]	1052 ± 780	177 ± 50[a,b]	935 ± 506
	S-NOR	R-NOR	S-NOR	R-NOR
t_{max} (min)	140 ± 85	126 ± 97	95 ± 50	73 ± 52
C_{max} (ng ml^{-1})	54 ± 16[b]	27 ± 11	107 ± 37[a,b]	51 ± 16[a]
AUC (μg min $ml^{-1}kg^{-1}$)	37.4 ± 22.5[b]	16.2 ± 13.3	72.4 ± 25.0[a,b]	30.5 ± 10.2[a]

[a] Value is significantly different from that of single dose.

[b] **Value is significantly different from that of R-enantiomer.**

Table 3.7 **Bioavailability of VER enantiomers after a 10 mg/kg oral dose to rats. Data are presented as mean ± SD (n = 5 per treatment).**

	Single Dose		Multiple Dose	
	S-VER	R-VER	S-VER	R-VER
Bioavailability (%)	17.6 ± 3.0[b]	6.6 ± 3.5	27.1 ± 8.0[a,b]	8.4 ± 2.4

[a] Value is significantly different from that of single dose.
[b] Value is significantly different from that of R-enantiomer.

Table 3.8 **AUC R/S ratios for VER, NOR and NOR/VER after a 10 mg/kg oral dose of racemic VER in rats. Data are presented as mean ± SD (n = 10 per treatment).**

	Single Dose		Multiple Dose	
	VER	NOR	VER	NOR
R / S Ratio	0.29 ± 0.14	0.41 ± 0.15	0.22 ± 0.66[c]	0.44 ± 0.11
	S	R	S	R
NOR / VER Ratio (%)	0.51 ± 0.12[b]	0.96 ± 0.65[a]	0.82 ± 0.33[a,b]	1.70 ± 0.71

[a] Value is significantly different from that of single dose.
[b] Value is significantly different from that of R-enantiomer.
[c] Value is significantly different from that of NOR.

Table 3.9 **Pharmacokinetic parameters of VER enantiomers after 1.0 and 2.5 mg/kg intravenous administration of racemic VER in rats. Data are presented as mean ± SD (n = 3 per group).**

	1.0 mg/kg		2.5 mg/kg	
	S-VER	R-VER	S-VER	R-VER
C_{max} (μg ml^{-1})	0.86 ± 0.17^{a,b}	0.41 ± 0.08^{a,c}	1.84 ± 0.63^{a,b}	1.26 ± 0.67^{a}
V_{ss} (l kg^{-1})	1.59 ± 0.26^{b}	2.74 ± 0.33	1.64 ± 0.20^{b}	2.98 ± 0.60
$t_{1/2}$ (min)	57.5 ± 21.3	94.7 ± 29.4	76.6 ± 23.9	91.6 ± 22
AUC (μg min ml^{-1} kg^{-1})	39.7 ± 6.4^{b,c}	32.9 ± 6.2^{c}	82.0 ± 5.4^{b}	59.5 ± 3.1
CL_s (ml min^{-1} kg^{-1})	35.4 ± 5.6^{b}	42.8 ± 7.2^{c}	42.0 ± 4.5^{b}	57.6 ± 5.2

[a] Calculated data by extrapolation.
[b] Value is significantly different from that of R-enantiomer.
[c] 1.0 mg/kg is significantly different from 2.5 mg/kg.

Table 3.10 Free fraction of VER enantiomers in the rat plasma at different plasma enantiomer concentrations (n = 3 per group).

ng/ml	S-VER	R-VER	S:R
2000	0.087 ± 0.006*	0.131 ± 0.003*	0.665 ± 0.067
1000	0.061 ± 0.012	0.107 ± 0.023	0.580 ± 0.094
500	0.051 ± 0.005	0.103 ± 0.032	0.530 ± 0.126
250	0.059 ± 0.004	0.114 ± 0.042	0.593 ± 0.193
125	0.060 ± 0.007	0.105 ± 0.024	0.617 ± 0.213

*Significant difference between the group and all the other groups (one tail unpaired t-test and one way ANOVA respectively).

Table 3.11 **CL_{int} (ml/min/g liver) values of VER enantiomers after constant infusion of racemic VER over a range of concentration (2.24 to 43.3 μM, n = 3 to 4 in each group).**

$C_{in\ (\mu M)}$	2.24 ± 0.03	2.97 ± 0.07	3.60 ± 0.05	5.80 ± 0.11	8.25 ± 0.29	12.45 ± 0.15	16.25 ± 0.44	43.30 ± 2.98
$CL_{int(S\text{-}VER)}$	31.23 ± 2.36[a]	22.98 ± 2.60	10.41 ± 3.79[a]	8.98 ± 2.26[a]	6.66 ± 0.97[a]	3.55 ± 0.87[a]	2.89 ± 0.72	3.15 ± 0.67
$CL_{int(R\text{-}VER)}$	23.28 ± 0.97	26.19 ± 2.78	21.71 ± 4.05	15.63 ± 4.67	9.73 ± 1.47	4.54 ± 0.64	3.43 ± 0.35	2.77 ± 0.67
S/R	1.34 ± 0.05	0.88 ± 0.06	0.47 ± 0.08	0.46 ± 0.13	0.69 ± 0.07	0.78 ± 0.10	0.84 ± 0.14	1.15 ± 0.11

[a] Value is significantly different from that of R-enantiomer based on a paired *t* test ($P<0.05$).

[b] The comparison across all groups calculated by ANOVA ($P<0.05$); the underlined values were not statistically different from each other.

Table 3.12 **The effect of VER pretreatment on kinetic parameters of VER in liver perfusion experiments (n = 6 per group).**

Kinetic parameters	Control	Verapamil pretreated
C_{in} (μM)	8.05 ± 0.26	8.13 ± 0.27
E	0.65 ± 0.21	0.57 ± 0.24
CL_{int} (ml/min/g liver)	8.60 ± 4.82	6.41 ± 4.70
C_{ss} (Nor)	0.80 ± 0.10	0.76 ± 0.20
C_{ss} (D-617)	0.47 ± 0.10	0.40 ± 0.11
C_{ss} (D-620)	0.22 ± 0.08	0.13 ± 0.06[a]
R/S ratio	0.70 ± 0.06	0.73 ± 0.01
T_{ss} (min)	60 ± 20	71 ± 8
MB (%)	53.70 ± 19.54	62.45 ± 19.01

[a] $P < 0.05$, Student's *t*-test.

Table 3.13 Kinetic parameters of VER and NOR enantiomers calculated during the washout period in stop infusion experiments (C_{in}: 43.3 ± 3.0 μM, n = 3).

	S-VER	R-VER	S-NOR	R-NOR
AUFC(nmol/g liver)	594 ± 26.9	652 ± 26.1	64.9 ± 7.5	46.6 ± 4.8
K_{tw}	70.9 ± 3.0	73.9 ± 4.0	33.3 ± 7.3	31.7 ± 5.5
$T_{1/2}$ (min)	15.0 ± 1.1	15.6 ± 2.3	26.8 ± 4.3	24.6 ± 2.4

Table 3.14 Inhibition of VER enantiomer metabolism in hepatocytes by LD, DZ, DPH and NOR[a].

	S-VER					
Inhibitor	Type of inhibition	Apparent K_m (μM)	Apparent V_{max} (pmol/min/10^6 cells)	Apparent K_i (μM)	Plasma Con. (μM)	Estimated inhibition *in vivo* (%)
LD	Competitive	7.51 ± 1.15	588 ± 99	150 ± 98.8	6.40~21.3[c]	4.08-12.4
DZ	Mixed	7.47 ± 0.47	597 ± 137	24.4 ± 2.75	0.18~0.36[d]	0.73-1.46
DPH	Mixed	7.21 ± 0.41	621 ± 37.2	62.2 ± 13.9	0.38~1.27[e]	0.61-2.00
NOR	Competitive	7.29 ± 0.54	574 ± 43.4	17.6 ± 1.98[b]	0.30~0.82[f]	1.67-4.44

	R-VER					
Inhibitor	Type of inhibition	Apparent K_m (μM)	Apparent V_{max} (pmol/min/10^6 cells)	Apparent K_i (μM)	Plasma Con. (μg/ml)	Estimated inhibition *in vivo* (%)
LD	Competitive	8.05 ± 1.32	540 ± 113	126 ± 15.1	6.40~21.3[c]	4.85-14.5
DZ	Mixed	8.01 ± 2.31	638 ± 133	22.5 ± 6.61	0.18~0.36[d]	0.79-1.57
DPH	Mixed	7.46 ± 0.85	555 ± 39.3	56.0 ± 6.98	0.38~1.27[e]	0.67-2.22
NOR	Competitive	7.89 ± 1.07	537 ± 45.7	24.3 ± 4.37[b]	0.30~0.82[f]	1.22-3.27

[a] Values are reported as mean ± SD (n=4).

[b] Statistical differences between the two enantiomers based on a paired student *t*-test.

[c] *Benowitz et al.*(1978).

[d] *Smith et al.* (1983); Kinney *et al.* (1981).

[e] *Glazko et al.* (1974).

[f] *Freedman et al.* (1981).

Table 3.15 **The effect of preincubation (0 or 10 min) of rat hepatocytes with several tertiary amines (25 μM) on the metabolic rates of VER enantiomers (VER concentration: 5 μM)^{a}.**

	Inhibition percentage (%)^{b}			
	S-VER		R-VER	
Inhibitor	0 min preincubation	10 min preincubation	0 min preincubation	10 min preincubation
LID	17.6 ± 5.96^{c}	32.7 ± 1.05	18.6 ± 3.71^{c}	37.6 ± 15.2
DZ	40.7 ± 10.02^{c}	80.8 ± 2.76	52.1 ± 7.22^{c}	82.6 ± 3.56
DPH	32.3 ± 8.55^{c}	56.5 ± 4.87	36.4 ± 11.1^{c}	60.3 ± 7.69
NOR	51.9 ± 15.5^{d}	61.0 ± 5.09^{c}	43.3 ± 7.47	45.03 ± 8.69

[a] Values are reported as mean ± SD (n=4).

[b] The control rates of metabolism are 97.6 ± 10.2 and 110.0 ± 17.9 nM/min/10^{6} cells for S-VER and R-VER respectively.

[c] Statistical differences between the two treatments based on unpaired student *t*-test.

[d] Value is significantly different from that of the R-enantiomer based on a paired student *t*-test.

Table 3.16 **The effect of preincubation (0 or 10 min) of rat hepatocytes with several tertiary amines (25 μM) on the formation rate of NOR enantiomers (VER concentration: 5 μM)[a].**

	Inhibition percentage (%)[b]			
	S-NOR		R-NOR	
Inhibitor	0 min preincubation	10 min preincubation	0 min preincubation	10 min preincubation
LID	2.01 ± 0.94[c]	10.7 ± 1.05	2.35 ± 1.71[c]	9.89 ± 2.10
DZ	35.7 ± 6.13[c]	52.0 ± 14.0	32.4 ± 5.34[c]	50.4 ± 8.52
DPH	~[d]	~[d]	~[d]	~[d]

[a] Values are reported as mean ± SD (n=4).

[b] The absolute formation rates of S-NOR and R-NOR are 14.3 ± 2.5 and 11.5 ± 2.2 nM/min/10^6 cells respectively.

[c] Statistical differences between the two treatments based on unpaired student *t*-test.

[d] No inhibition of NOR formation could be detected.

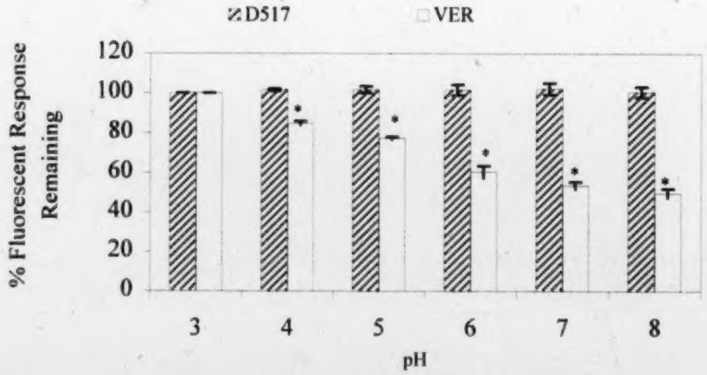


Figure 3.1 Fluorescent responses of 500 ng/ml VER and D-517 at pH 3-8. Results are compared with these at pH 2.2 (mean ± SD, n=4). *Significantly different from the fluoresecent response of VER at pH 2.2 (p<0.05).

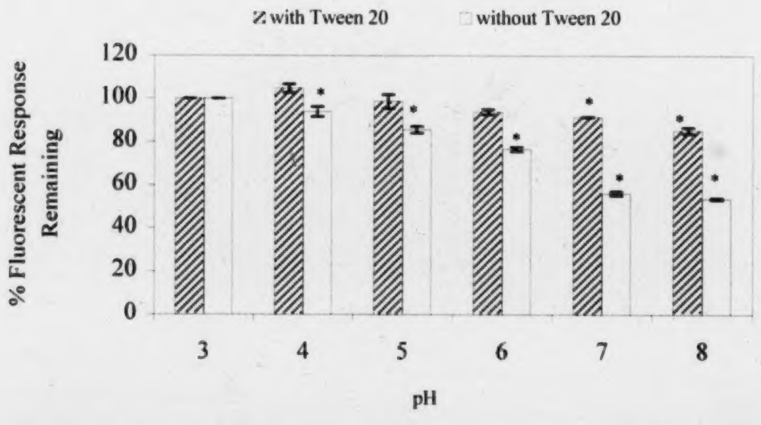


Figure 3.2 pH dependent fluorescent response of 500 ng/ml VER with and without 1.75% Tween 20 (mean ± SD, n=4).
***Significantly different from the fluoresecent response of VER at pH 2.2 (p<0.05).**

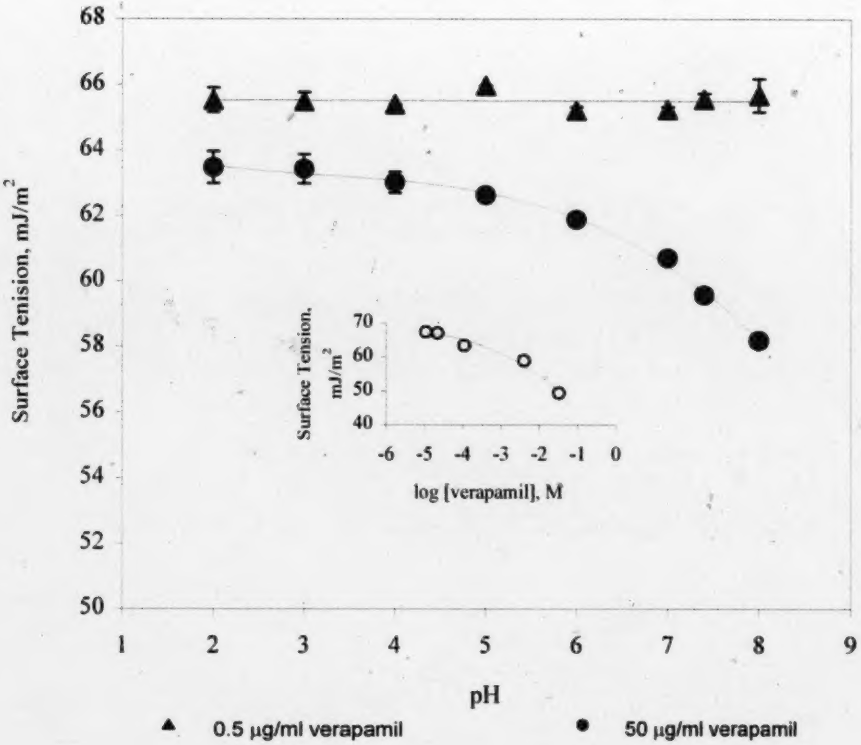


Figure 3.3 Dependence of surface tension of VER on pH . Data are mean ± SD (n=5). Lines represent model-fitted linear and biexponential decline curve. The inset shows the dependence of surface tension of verapamil on concentration at pH 5.6.

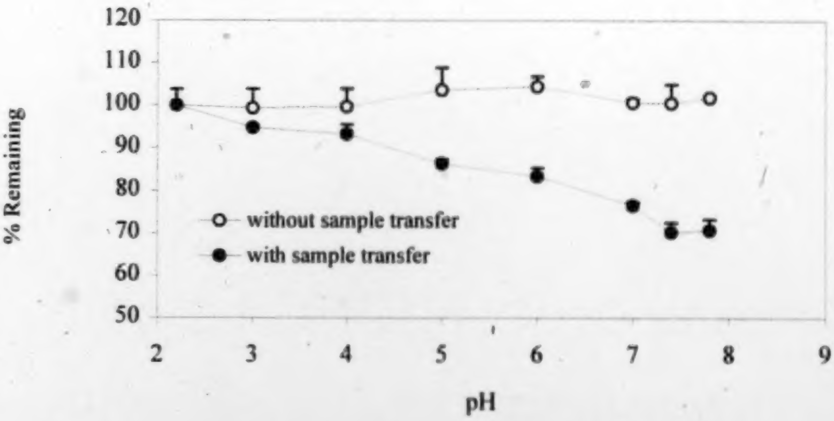


Figure 3.4 pH dependent recovery of ^{3}H-VER with or without the use of pipette tip to transfer. Radiometric method was used for this measurement (mean ± SD, n=3).

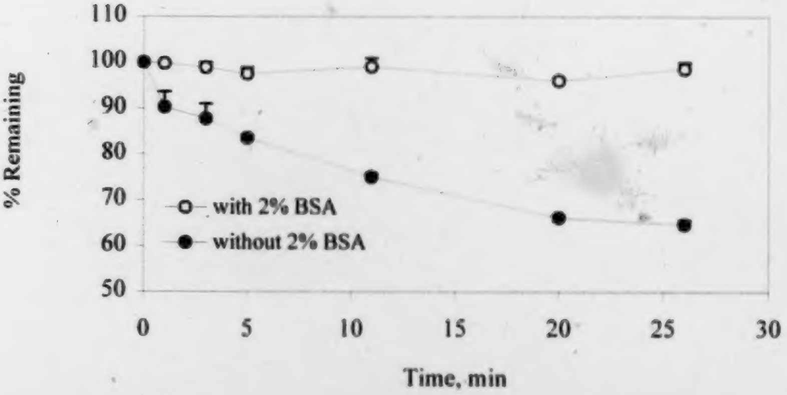


Figure 3.5 Stability of VER in polypropylene pipette tips with and without 2% BSA measured using radiometric method (mean ± SD, n=3).

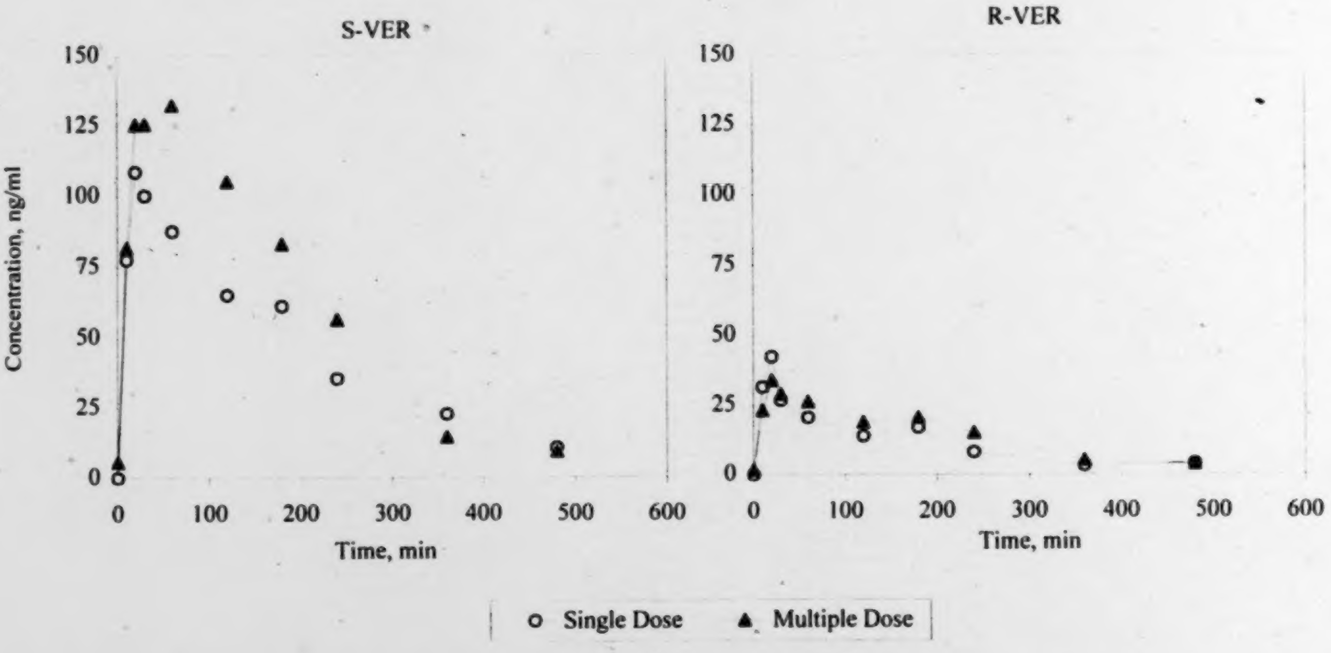


Figure 3.6 The plasma concentration-time profiles of S-, and R-VER after single and multiple administration of 10 mg/kg racemic VER. Values are shown as the mean of ten animals. (For clarity, only mean values are depicted.)

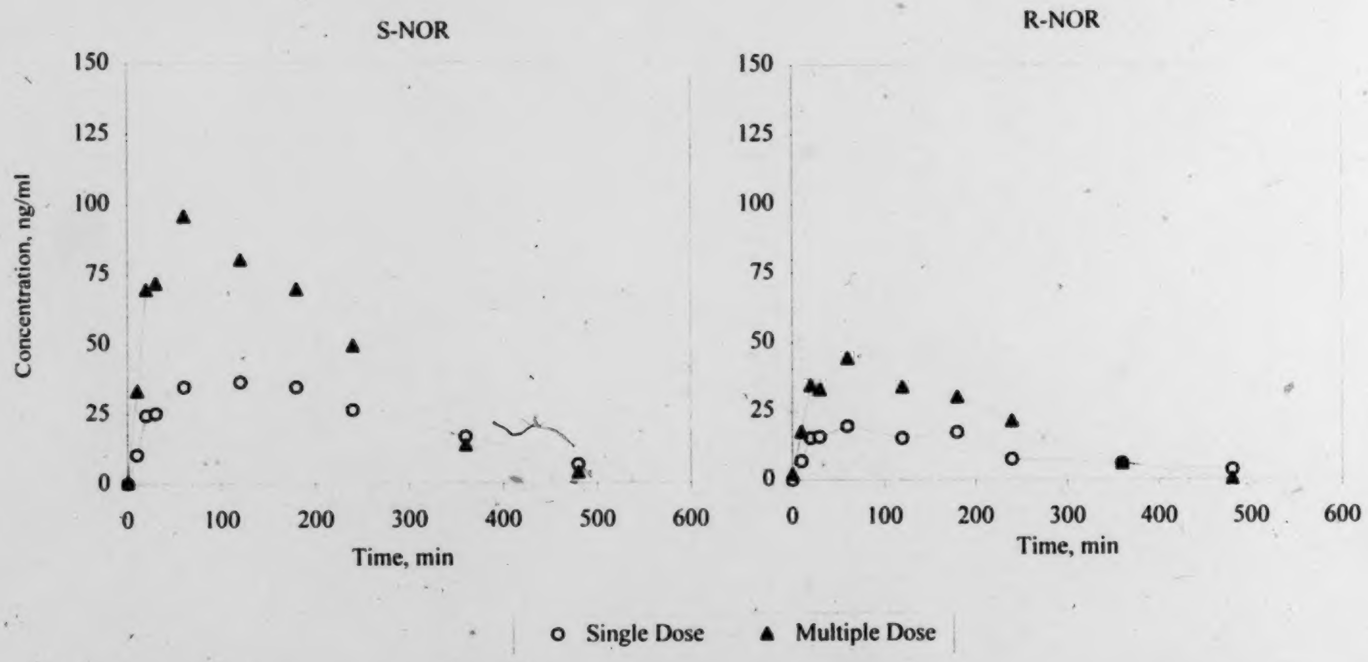


Figure 3.7 **The plasma concentration-time profiles of S-, and R-NOR after single and multiple administration of 10 mg/kg of racemic VER. Values are shown as the mean of ten animals. (For clarity, only mean values are depicted.)**

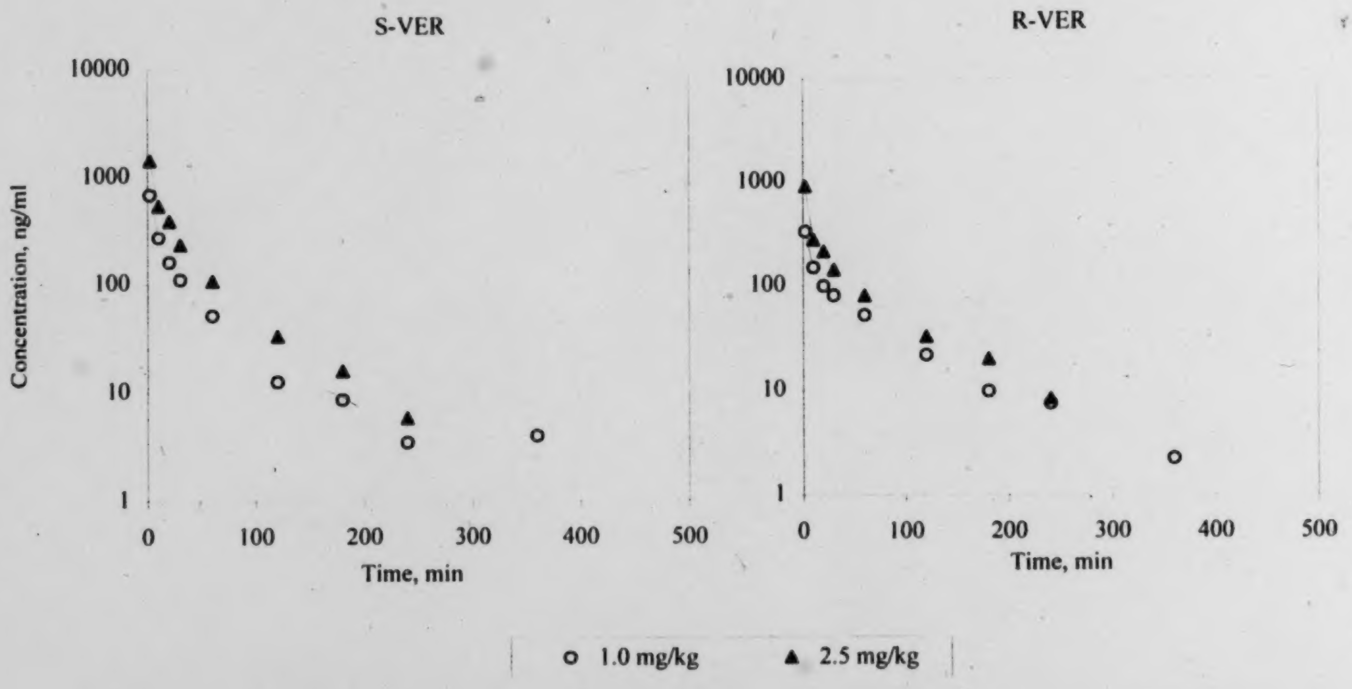


Figure 3.8 **The average plasma concentration-time profiles of S-, and R-VER after i.v. administration of 1.0 and 2.5 mg/kg racemic VER. Values are shown as the mean of three animals. (For clarity, only mean values are depicted.)**

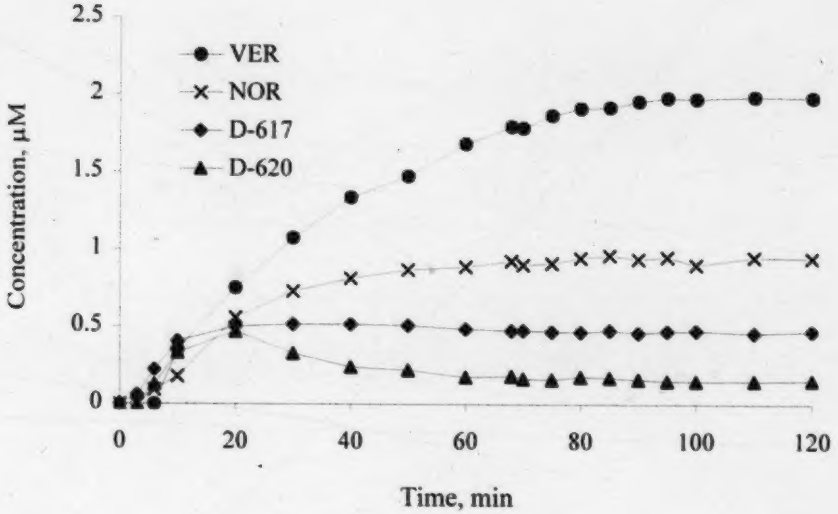


Figure 3.9 Representative concentration *vs.* time profiles of VER and its metabolites in the effluent of a perfused rat liver. C_{in} = 8.34 μM

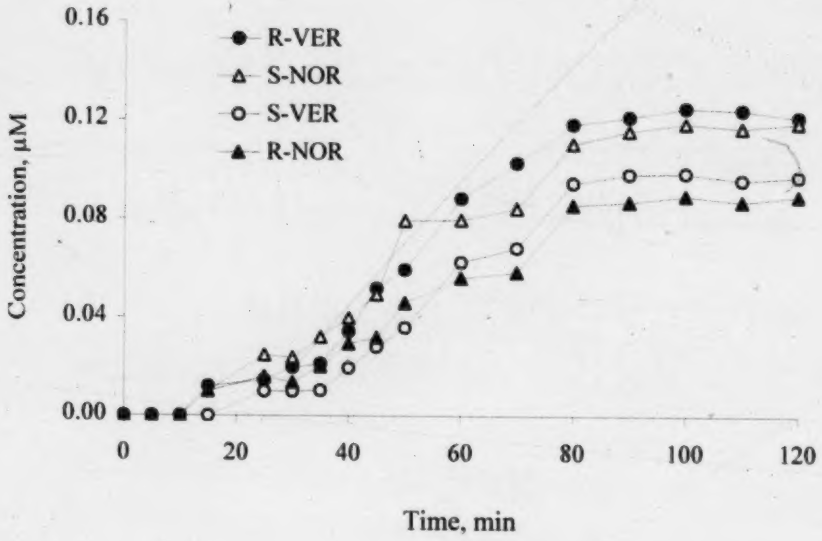


Figure 3.10 Representative concentration *vs.* time profile of S-and R-enantiomers of VER and NOR in the effluent of a perfused rat liver. C_{in} = 2.2 µM

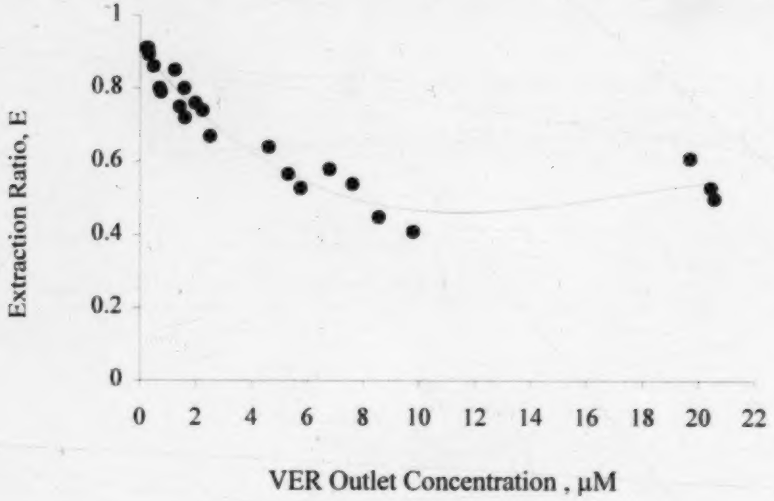


Figure 3.11 Plot of steady-state extraction ratio *vs.* effluent VER concentration.

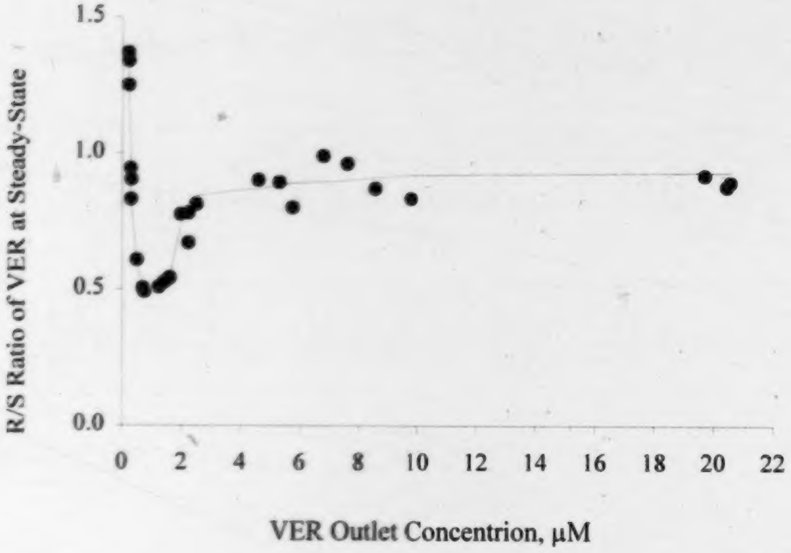


Figure 3.12 Plot of the ratio of R- to S-VER *vs.* effluent VER concentration.

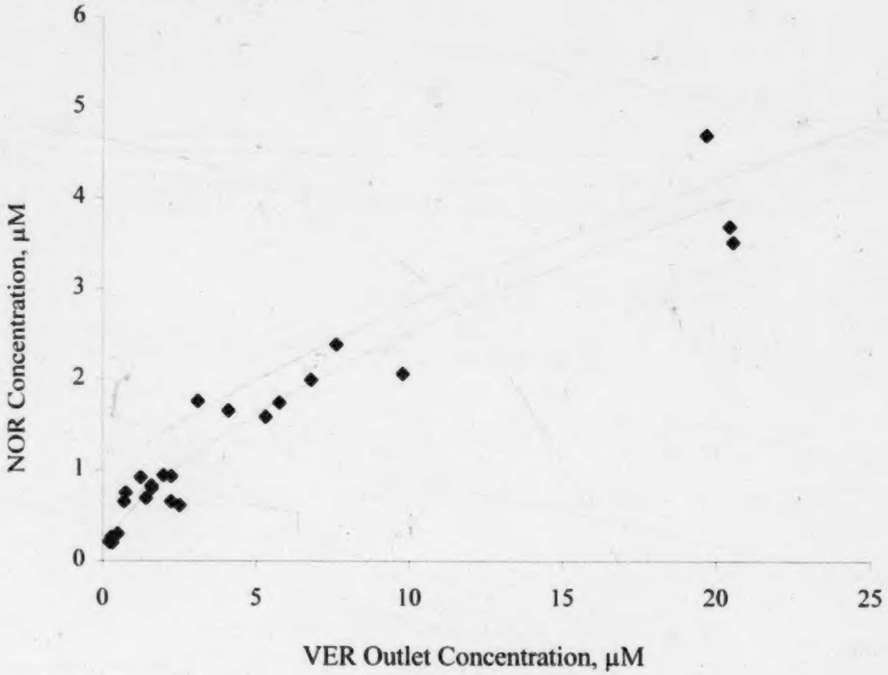


Figure 3.13 Plot of steady-state NOR *vs.* effluent VER concentration at steady-state.

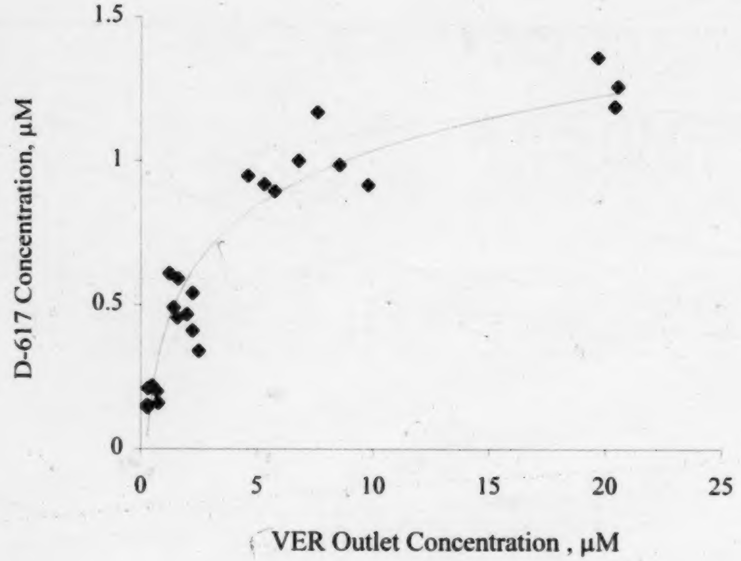


Figure 3.14 Steady-state D-617 concentrations *vs.* VER outlet concentration at steady-state.

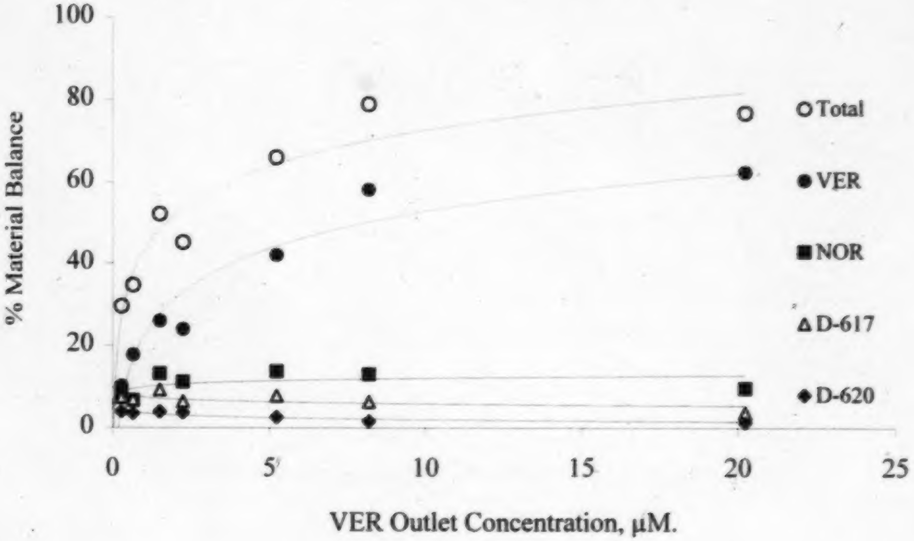


Figure 3.15 VER outlet concentrations *vs.* mean of material balance at steady-state.

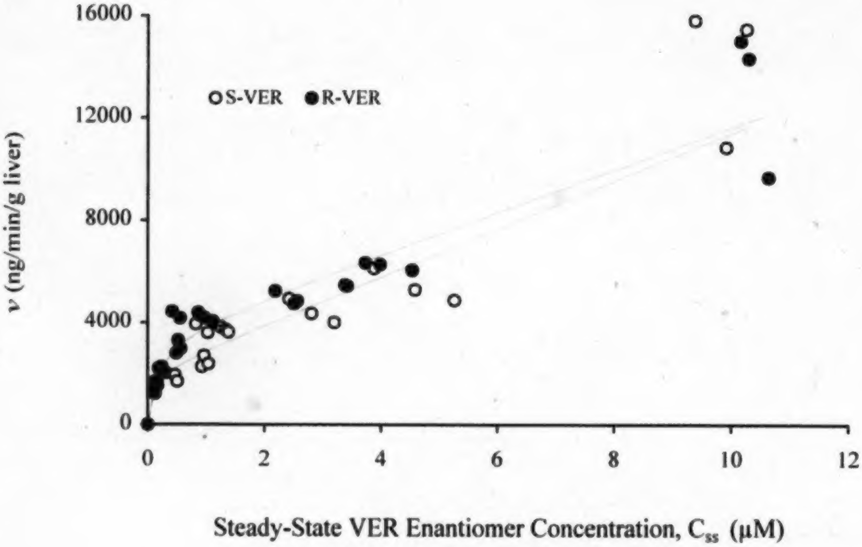


Figure 3.16 Metabolic rate of S-VER (●) and R-VER (○) *vs.* steady-state concentration of each enantiomer fitted with a mixed elimination model: $v = CL_2C_{ss} + (V_{max} * C_{ss}/K_m+C_{ss})$.

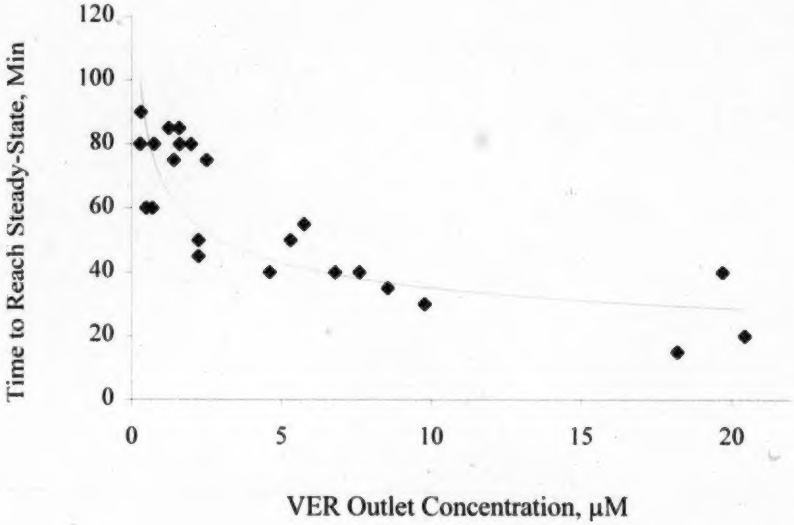


Figure 3.17 Time to reach steady-state *vs.* VER outlet concentration at steady-state.

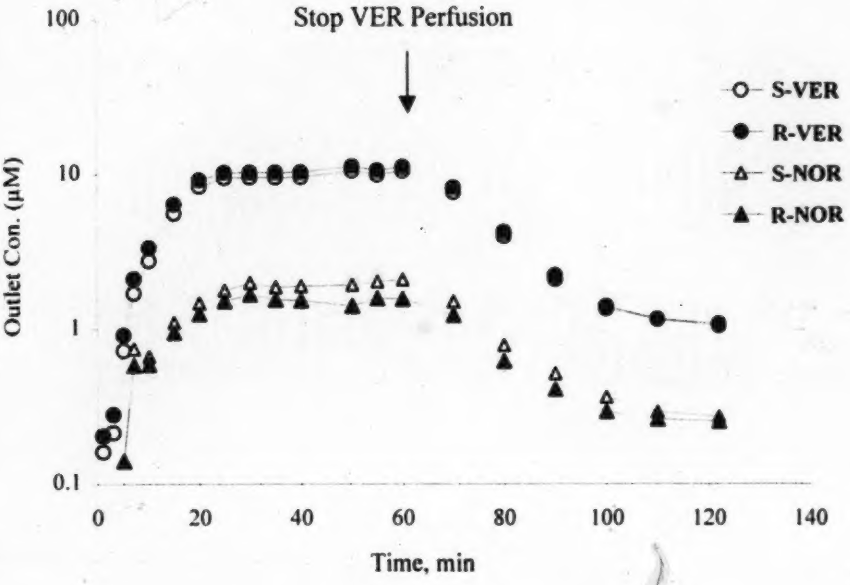


Figure 3.18 Representative washout profiles of S-, R-VER and NOR. (C_{in} = 44 μM)

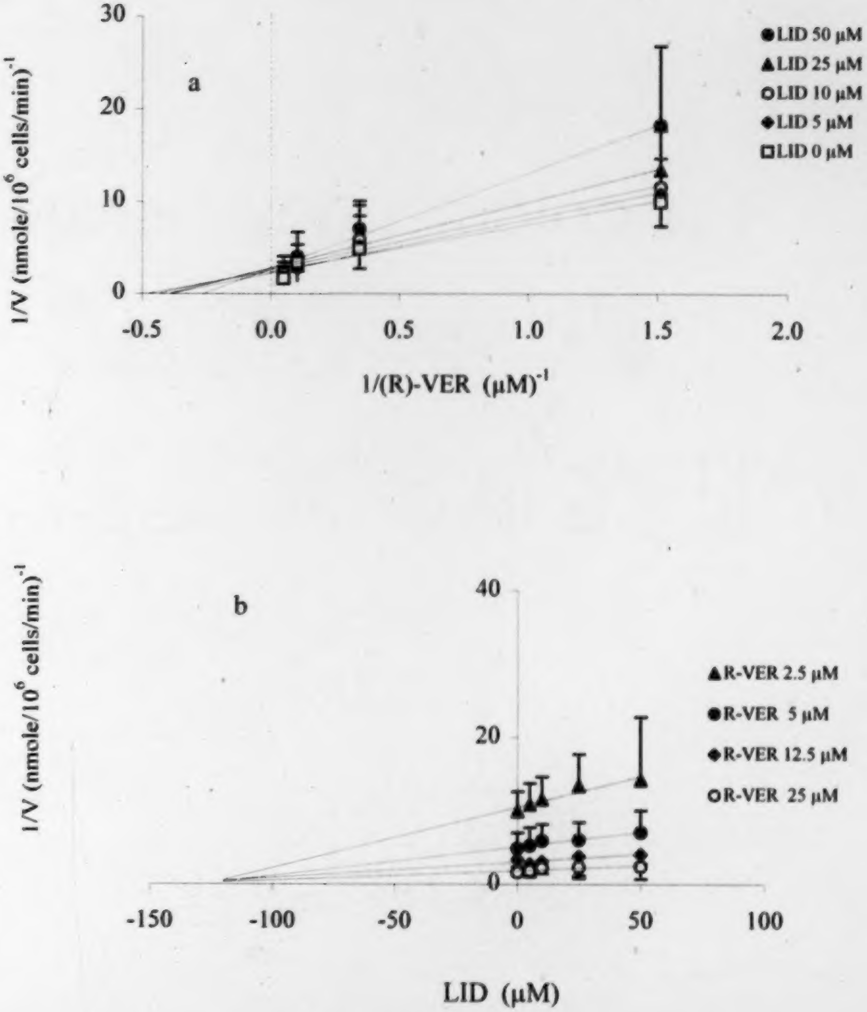


Figure 3.19 Lineweaver-Burke (a) and Dixon (b) plots showing the effect of LID on the disappearance of R-VER in the hepatocyte system. Each point represents the mean ± SD value obtained from four separate experiments and lines were determined by linear regression.

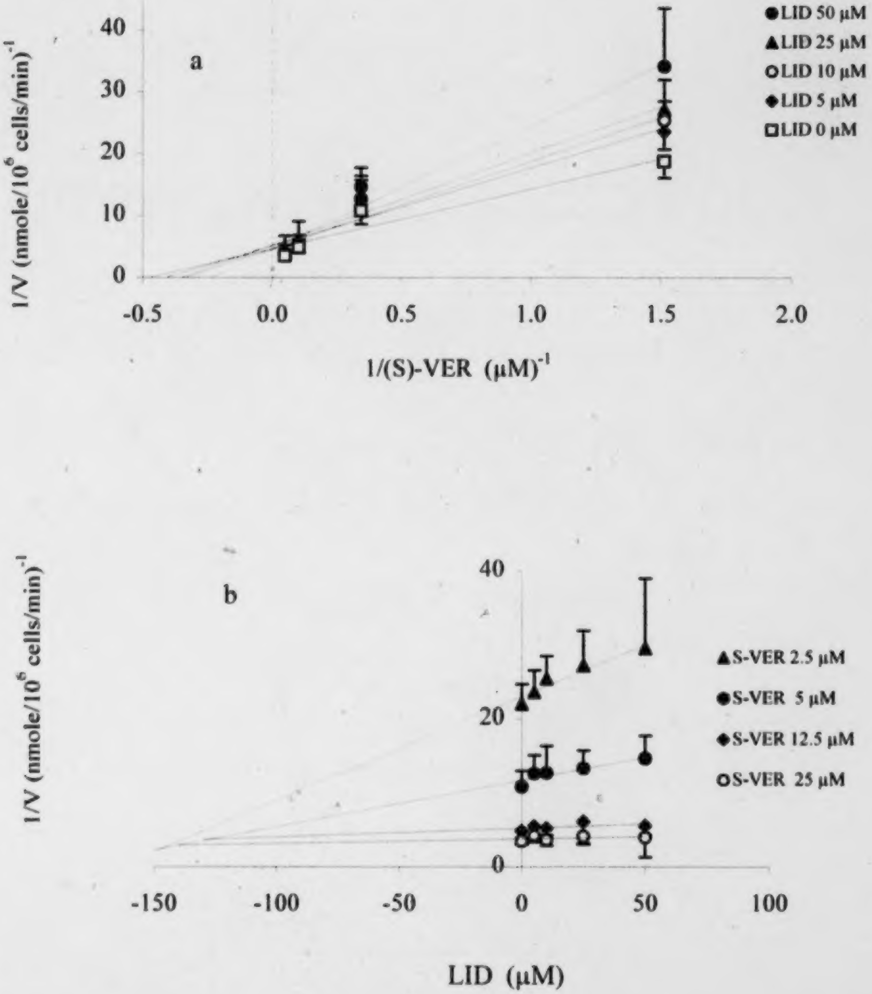
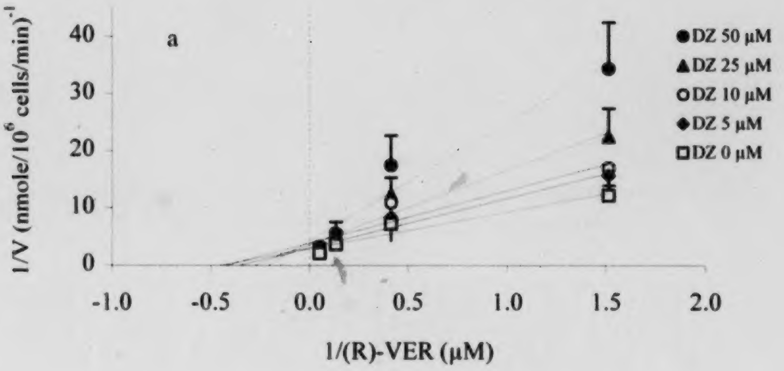


Figure 3.20 Lineweaver-Burke (a) and Dixon (b) plots showing the effect of LID on the disappearance of S-VER in the hepatocyte system. Each point represents the mean ± SD value obtained from four separate experiments and lines were determined by linear regression.



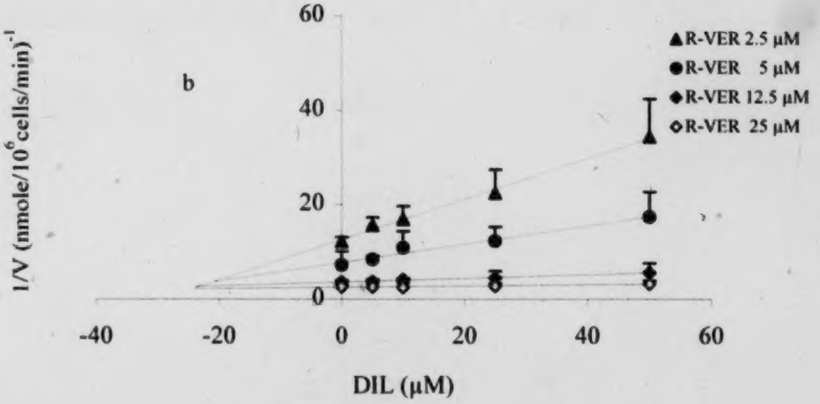


Figure 3.21 Lineweaver-Burke (a) and Dixon (b) plots showing the effect of DZ on the disappearance of R-VER in the hepatocyte system. Each point represents the mean ± SD value obtained from four separate experiments and lines were determined by linear regression.

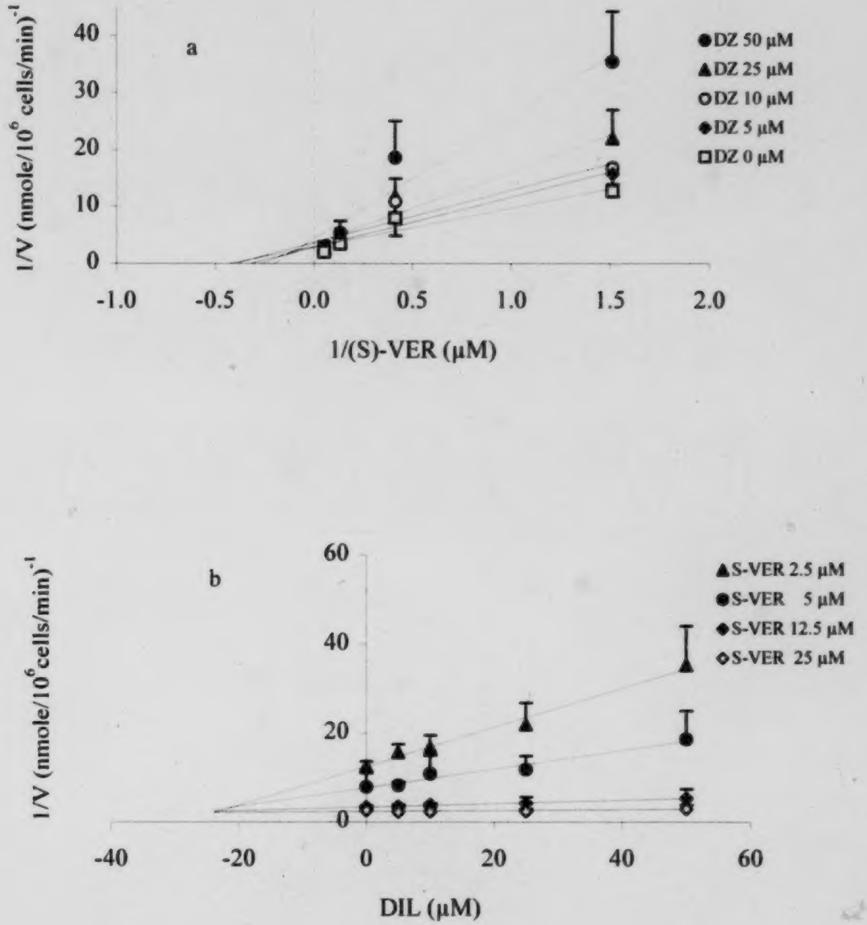


Figure 3.22 Lineweaver-Burke (a) and Dixon (b) plots showing the effect of DZ on the disappearance of S-VER in the hepatocyte system. Each point represents the mean ± SD value obtained from four separate experiments and lines were determined by linear regression.

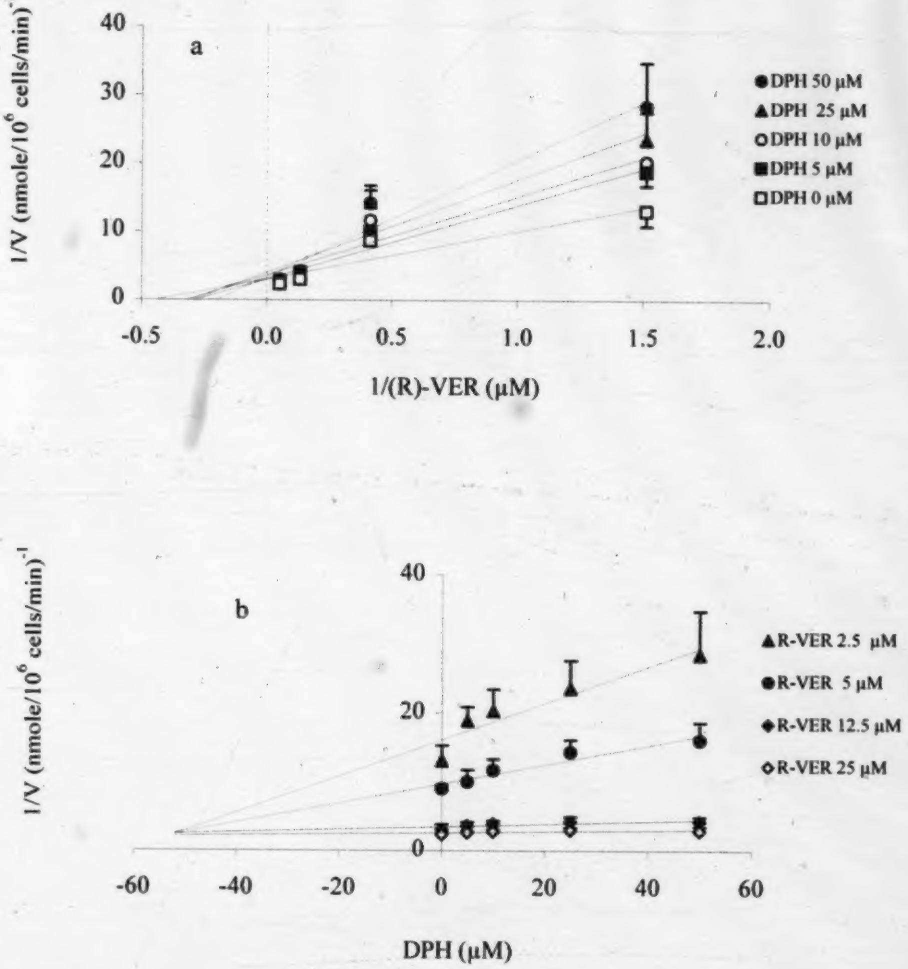


Figure 3.23 Lineweaver-Burke (a) and Dixon (b) plots showing the effect of DPH on the disappearance of R-VER in the hepatocyte system. Each point represents the mean ± SD value obtained from four separate experiments and lines were determined by linear regression.

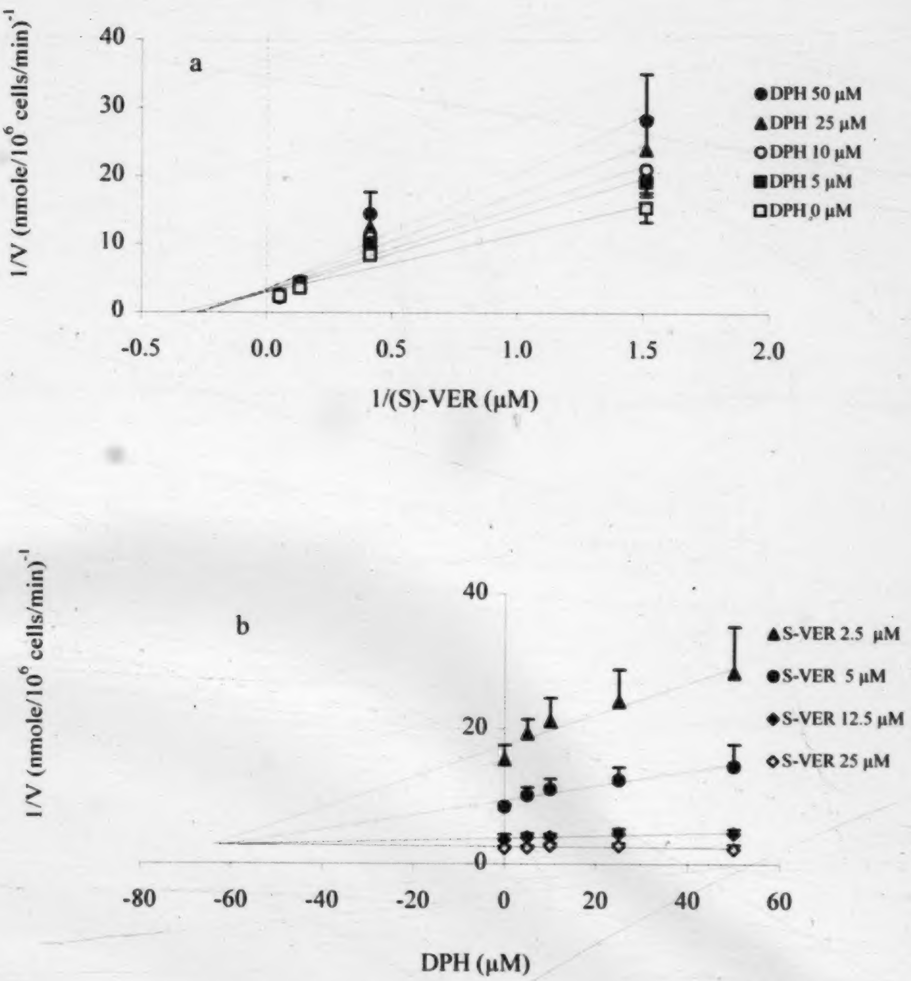


Figure 3.24 Lineweaver-Burke (a) and Dixon (b) plots showing the effect of DPH on the disappearance of S-VER in the hepatocyte system. Each point represents the mean ± SD value obtained from four separate experiments and lines were determined by linear regression.

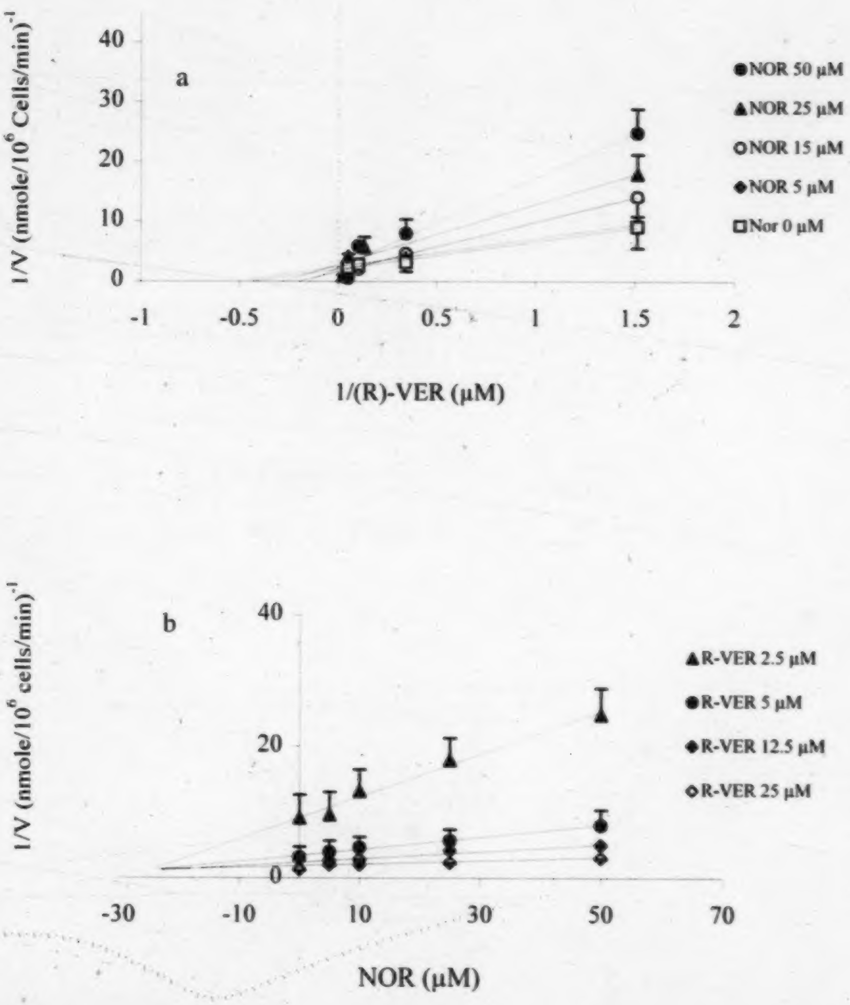


Figure 3.25 Lineweaver-Burke (a) and Dixon (b) plots showing the effect of NOR on the disappearance of R-VER in the hepatocyte system. Each point represents the mean ± SD value obtained from four separate experiments and lines were determined by linear regression.

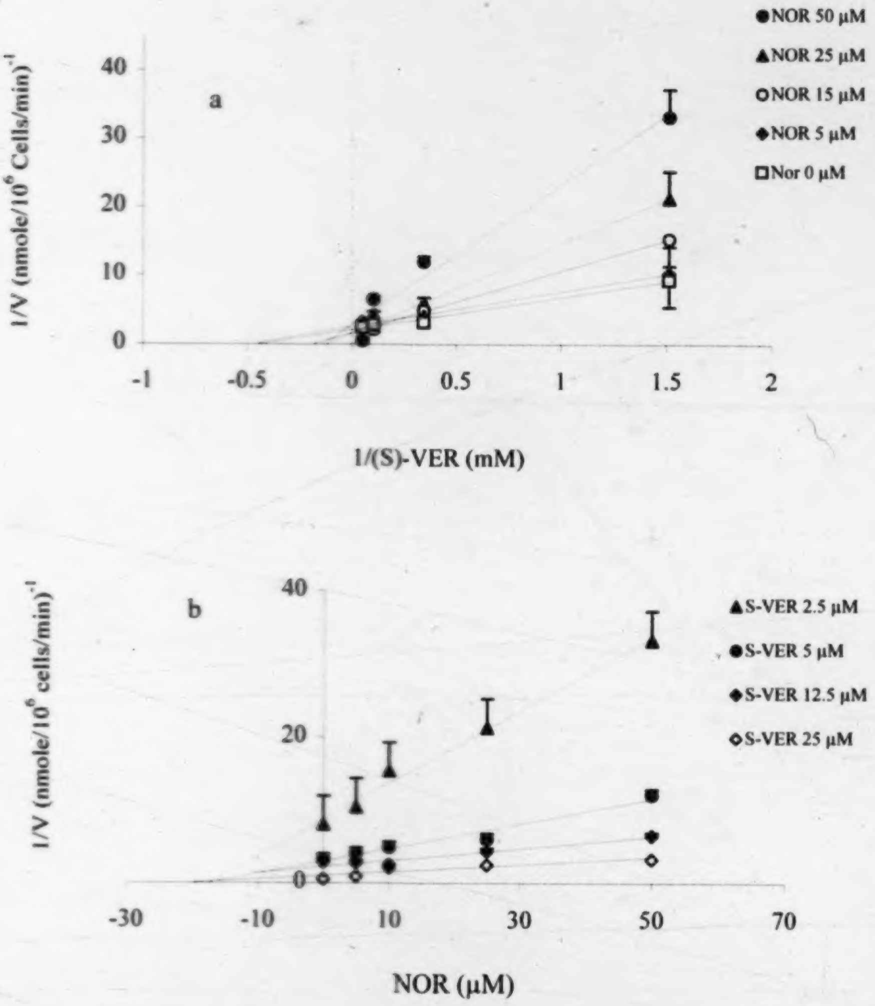


Figure 3.26 Lineweaver-Burke (a) and Dixon (b) plots showing the effect of NOR on the disappearance of S-VER in the hepatocyte system. Each point represents the mean ± SD value obtained from four separate experiments and lines were determined by linear regression.

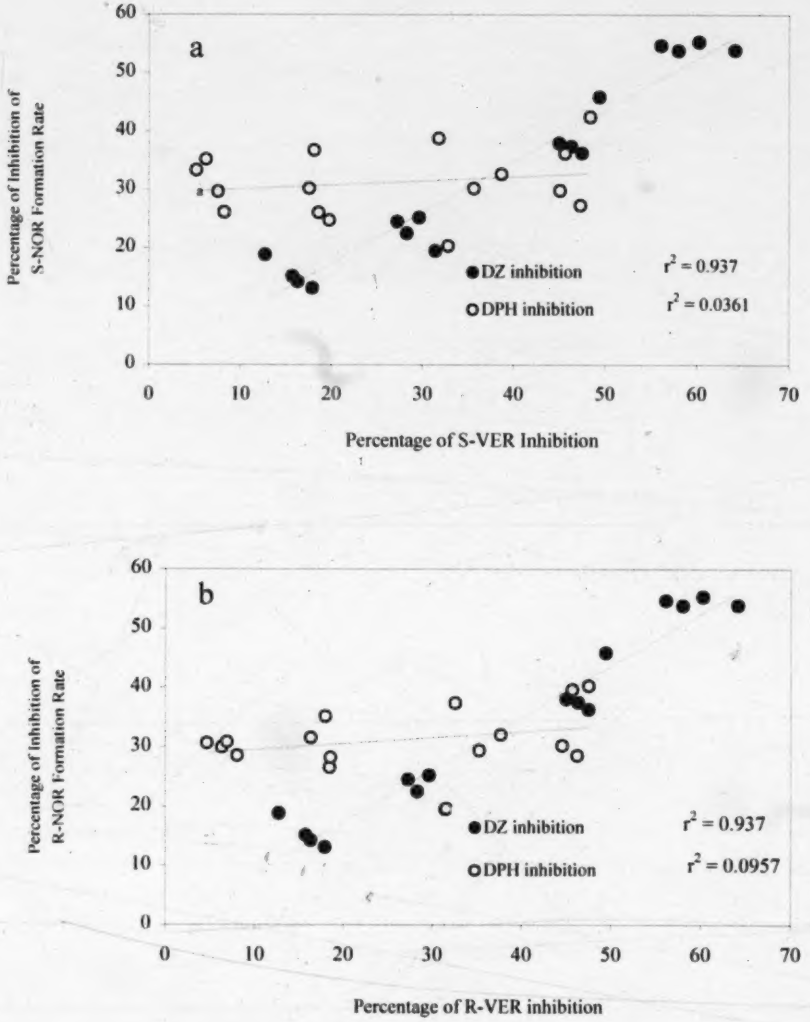


Figure 3.27 Correlation between inhibition of NOR formation and inhibition of VER disappearance and the effects of DIL and DPH. The concentration of VER was 5 μM and the concentration of DZ and DPH was 5, 10, 25, 50 μM. (a) S-enantiomer of VER and NOR; (b) R-enantiomer of VER and NOR.

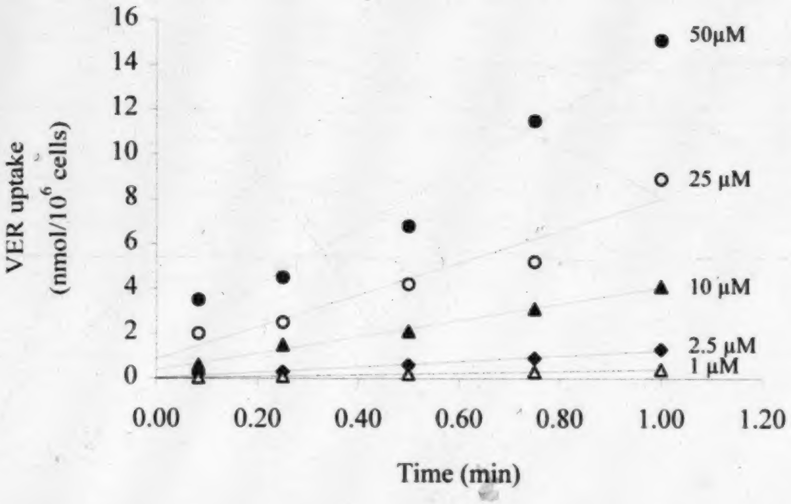


Figure 3.28 Uptake of VER by cultured hepatocytes at differenct VER concentrations (1 to 50 µM). The data shown are of a representative experiment. Initial uptake velocity for each substrate concentration was obtained from the slope of the regression line through the data points.

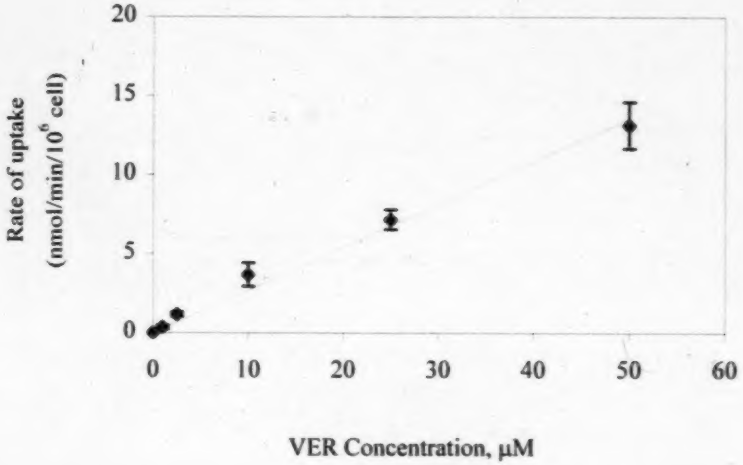


Figure 3.29 Uptake rates for VER *vs*. VER concentrations in incubation medium.

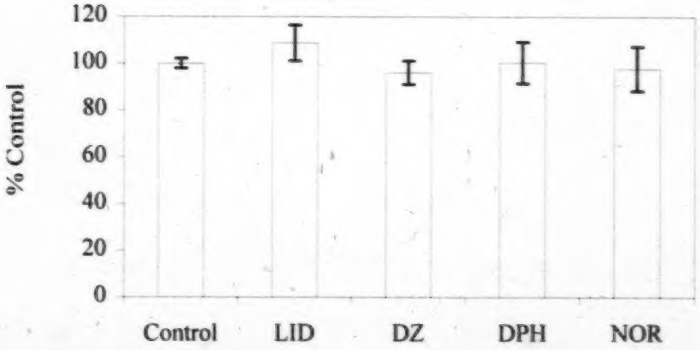


Figure 3.30 Effect of the tertiary amines on VER uptake velocity. VER concentration was 50 µM; All tertiary amine concentrations were 200 µM. Each point represents the mean and SD of three experiments.

4. DISCUSSION

4.1 Identification of pH dependent pipette tip adsorption

In this study, citric-phosphate buffer solution covering the pH range from 2.2 to 8.0 with a constant ionic strength was used. It was reported that the optimum pH for the chemical stability of VER was between the range of 3.2 to 5.6 (*Gupta 1985*). However, a pH dependent change in fluorescence was obvious over this pH range. The decreased fluorescent intensity of VER with increased pH from 2.2 to 8.0 resulted in a loss of ~ 40% VER during sample analysis (Figure 3.1). Solubility change and chemical degradation were ruled out. Furthermore, this pH dependent observation was not anticipated, since all the samples with different pH were basified with 1N NaOH (pH 13) before extraction. Thus, the same amount of neutral form would be extracted into the organic phase no matter what the original pH of the sample was unless there was a slow ionization conversion during the extraction. However, leaving the alkalinized samples for extended period did not change the outcome, indicating a slow process was not involved. Tween 20 partially decreased the pH dependent effect (Figure 3.2), suggesting interfacial phenomena might play a significant role. Lin (*1987*) has obtained similar results for diazepam. It was reported that pluronic surfactants could effectively prevent the adsorption of diazepam onto polyvinyl chloride bags.

Interfacial tension experiments confirmed that VER was surface active. Nonspecific adsorption will occur if the solute is surface active. The result that the surface tension decreased by 10% when pH was increased from 3.0 to 8.0 at 50 μg/ml VER solutions indicated that deprotonation increases surface stability, with the accumulation of VER at the surface increasing at alkaline pH. In addition, changes in the pH of the aqueous phase usually cause marked changes in the adsorption of ionic surfactants onto charged solid substrates. As the pH of the aqueous phase is raised, a solid surface will usually become more negative, or less positive, with a consequent increase in the adsorption of cationics (*Connor and Ottewill 1971, Van Senden and Koning 1968*). A surfactant can compete with VER to reduce interfacial free energy in the sample interfaces, and hence reduce the tendency of VER toward the interface and increase the stability of VER in the bulk solution.

A radiometric method was used to identify adsorption. It was found that surface adsorption of VER to pipette tips accounted for the pH dependent quantification differences observed in fluorescent detection. During the sample transfer process, a significant amount of VER was left behind on the walls of the pipette tip and the amount of loss was pH dependent. The adsorption could be eliminated if the solution contained 2% BSA. It was reported that nonspecific adsorption of taxol to containers could be totally circumvented by using fetal bovine serum (FBS) (*Song et al. 1996*). However, the mechanism of this protein effect is not clear. It was proposed that the "reservoir effect" of the protein-drug complex was responsible for the elimination of drug non-specific adsorption (*Connor et al. 1971*).

The interaction between a surface and adsorbed species may include hydrogen bonding, hydrophobic binding or van der Walls forces (*Parfitt and Rochester 1983*). The material of the tested pipette tip is polypropylene. In the preliminary study, another brand name of pipette tips (**Fisher**brand® Tips, FisherScientific, Ottawa, ON, Canada) made of polypropylene showed the same degree of pH dependent change in fluorescent response.

The mechanism of the interaction of adsorption of surfactants and polymers is still not well understood and may be identified by studying the adsorption isotherm. In addition, it is interesting to note that although both Sarstedt® tubes and pipette tips are made of polypropylene, only the latter shows significant adsorption. Since the properties of a polypropylene product may vary depending on the manufacturing process, such as the addition of specific additives, and surface treatments (*Giles and Pecina 1990*), it is speculated that these two kinds of polypropylene may exist in different forms and with different adsorptive capacities. The fact that the contact surface area per ml solution of pipette tip is approximate 2 times of that of the Sarstedt® tube may also be a contributing factor.

4.2 Rat *in vivo* studies

4.2.1 Single *vs.* multiple oral studies

The pharmacokinetics after single and after multiple oral dosing of racemic VER were compared. There were significant increases in AUC and oral bioavailability and a decrease in oral clearance of S-VER, while the kinetic parameters for R-VER didn't change significantly in rats after multiple dosing (Table 3.6). The changes in S-VER will not be due to accumulation from the previous doses since 70% of the plasma concentrations at the last sampling time (480 min) were below the quantifiable limit of 5ng/ml (Table 3.2). Therefore, the AUC calculated from time 0 to 480 min after single dose was equivalent to the value from time 0 to infinity, and the high AUC after multiple dosing was not due to accumulation from previous doses. Furthermore, the trough concentrations of S-VER (at 0 min for the last dose) were undetectable in 70% of the rats after repetitive oral VER administration (Table 3.4). It is unlikely that the contribution from the previous plasma concentration is a major factor responsible for the changes in S-VER. The results indicate that stereoselective time-dependent kinetics occurs in the rat model. In human, oral clearance of VER has been found to be reduced by 40% after 16 days of chronic dosing (*Eichelbaum and Somogyi 1984*). This time-dependent kinetics has been found for both S- and R-VER (*Gupta et al. 1996*). The patterns of plasma enantiomer ratio profiles differed substantially between single and multiple doses in human, indicating stereoselectivity changes with chronic dosing (*Longstreth 1993*). However, to what extent a reduced oral clearance of racemic VER in man is related to changes in the oral clearance of the individual VER enantiomers is not known. In rats, the decreased oral clearance of S-VER caused about 25.5% decrease in R/S-enantiomer ratio after multiple dosing. The AUC of NOR after multiple dosing was significantly increased, 93.7% for S-NOR and 88.7% for R-NOR. In addition, the NOR/VER ratio after multiple dosing was significantly increased, 63.3% for S-VER and 80.1% for R-VER indicating accumulation of NOR. An accumulation of NOR has been found in human (*Kates et al. 1981 and Frishman et al. 1982*). Two possibilities could have occurred – either the rate of elimination of NOR was reduced or the rate of its formation was enhanced or both. The decrease of VER clearance suggests that the N-dealkylation of NOR might be inhibited. Accumulation of NOR could either be the result of the same mechanism that caused the decrease in elimination of VER, or the cause of the decrease in elimination of VER. Higher NOR levels might inhibit the metabolism of its parent drug VER through product inhibition. The possibility of product inhibition will be discussed in section 4.4.3.

At this point, the exact mechanisms of time-dependent kinetics of S-VER are unknown. Increased AUC after multiple dosing can be a result of an increase in absorption, a decrease in plasma free fraction or in hepatic intrinsic clearance. VER has been shown to be well absorbed following oral administration (*Eichelbaum et al. 1981*). Complete gut absorption (F_g = 0.98) was observed by Hoffman *et al.* (*1995*) by using absorption probes with portal blood sampling. Although it has been found that VER is secreted by P-glycoprotein in the rat jejunum, its net absorption mainly occurs in ileum where an efflux transport system is lacking (*Saitoh and Aungst 1995*). Thus, it is unlikely that gut will contribute to the nonlinear kinetics of VER. A plasma protein-binding study found no change in plasma unbound fraction in the concentration range studied. Moreover, *Johnson* and *Akers* (*1995*) found no change in protein binding of R- and S-VER following single and multiple doses of racemic VER as compared with protein binding in pre-dose samples and there was no effects of VER metabolites on the R- and S-VER plasma protein binding. So, the time dependent kinetics of VER would not be related to plasma protein binding. The decreased hepatic intrinsic clearance, however, could be related to saturation of enzymes, enzyme inactivation, product inhibition, and saturation of binding to hepatic tissue. More detailed *in vitro* studies are required to elucidate the factors responsible for the variation the kinetics. They are discussed in section 4.3.

4.2.2 Intravenous studies

Caution may be needed for the interpretation of the i.v. results. Since oral dosing was the prime objective of this study, the i.v. study was performed after the single or multiple oral dose studies. Although a 7-day washout period was allowed, the i.v. data might be biased by the carryover effect from the oral treatment. However, this possibility is unlikely because the clearance data found (Table 3.9) are higher than those reported earlier in the literature (*Bhatti et al. 1997*), suggesting that the 7-day washout is sufficient to bring the metabolism back to normal and the data provides a good estimate of the i.v. VER kinetics.

The systemic clearance of R-VER (CL_s) increased significantly when the i.v. dose was increased from 1.0 to 2.5 mg/kg (Table 3.9). Since the volume of distribution was not affected by dose, an increase in elimination would be the major cause of this increase in Cl_s. Owing to the high hepatic extraction ratio of VER (S-VER: 0.82 and R-VER: 0.93), CL_s is predominantly dependent on the hepatic blood flow rate. R-VER has a higher extraction ratio than its antipode (Table 3.7), thus it has been more affected. Studies have shown that VER concentrations correlate closely with the changes in blood flow (*Neugebauer 1978 and Woodcock et al. 1981*). Therefore, a higher dose of VER could trigger an increase in hepatic blood flow and thus an increase in CL_s. The increase in CL_s with an increase in the intravenous dose could be a reflection of a dose dependent increase in hepatic blood flow.

A human study conducted by Meredith *et al.* (*1985*) has shown that acute administration of VER resulted in a significant increase in apparent liver blood flow, which fell significantly with continued administration. This study suggested that the accumulation of VER during chronic therapy was an apparent accumulation, and that the change in kinetic parameters was more appropriately described as a shortening of elimination half-life and an increase in clearance following acute dosing. However, based on the venous equilibrium model for hepatic clearance, changes in hepatic blood flow would have no net effect on the AUC after oral administration, i.e. the apparent oral clearance (*Pang and Rowland 1977*). Based on the i.v. data in rats, it can be speculated that the time-dependent reduction in S-VER clearance may not be due to the changes in hepatic blood flow, otherwise R-VER will be more effected than S-VER.

4.2.3 Comparison with the kinetics in humans

Close examination of the human and the rat data reveals that there are numerous similarities in the pharmacokinetic characteristics among the two species. Similar to human (*Vogelgesang 1984*), the intravenous dose resulted in a much lower difference in AUC between the two enantiomers (R/S: 0.83 ± 0.04 at 1mg/kg) when compared to that after oral administration (R/S: 0.29 after single dose). These results are consistent with the extensive stereoselective first-pass metabolism of VER in humans. Similar to human (*Gross et al. 1988)*, volume of distribution at steady state for the enantiomers of VER exhibited stereoselectivity in the rat. The V_{ss} values of R-VER reported in this study were between 42% to 45% higher than those of S-VER (Table 3.9). This difference is consistent with an approximately 50% higher free fraction of R-VER (Table 3.10 and *Laethem et al. 1994*). Thus, the larger volume of distribution of R-VER can be totally accounted for by its higher free fraction in plasma. The same rationale has been used to explain the higher volume of distribution of S-VER in human where the free fraction of S-VER is higher than R-VER. The major difference between the two species is the opposite stereoselectivity, which is consistent with others reports (*Bhatti et al. 1997 and Mehvar et al. 1994*). It was suggested that the different stereoselectivity observed in the two species might be due to structural differences in the metabolic enzymes and/or due to free fraction of the drug (*Nelson et al. 1988*). Our data have shown that both the systemic and oral clearances of R-VER were significantly higher than those of S-VER in the rat. In human, however, the S-enantiomer was found to have an oral clearance approximately 3 to 4 times greater than that of the R-enantiomer (S-VER: 7893 ml/min, R-VER: 2408 ml/min, *Kroemer et al. 1992*). However, the stereoselectivity of the oral clearance was mainly due to the 2-fold difference in the unbound fraction of VER in plasma between both forms (Gross *et al.* 1988). Only 1.3-fold difference was observed between the S- and R-VER intrinsic clearance in humans (S-VER: 8.72 ml/min/g, R-VER: 7.05 ml/min/g, *Iwatsub et al. 1997*). Mehvar and Reynolds (*1995*) studied the influence of protein binding on the direction of stereoselectivity in the kinetics of VER using isolated perfused rat livers. It was found that the direction of stereoselectivity in the first pass metabolism was mainly governed by the stereoselectivity in the protein binding of the drug. Without protein binding, the free intrinsic clearance of S-VER was similar to or slightly higher than its antipode.

4.3 Liver perfusion studies

In this study we found that the time-dependent change in VER metabolism observed in humans can be replicated in the isolated perfused rat liver system. A slow approach to C_{ss} for both metabolites and parent drug and a changing hepatic extraction with infusion time were prime indicates (Figure 3.9). Mehvar *et al.* (*1994*) found that perfused rat liver showed time-dependent kinetics, but no mechanistic results were reported. Based on the present data, the mechanism of the time-dependent kinetics may be largely attributed to saturable enzyme activity and reversible binding of VER to liver tissues.

4.3.1 Saturable kinetics of verapamil

The most dramatic changes in E values (Figure 3.11) and in stereoselectivity (Figure 3.12) occurred at low C_{out}. These changes tended to reach a plateau at higher C_{out} of VER (~ 4 μM), indicating the saturation of some tissue binding sites and/or completion of a slow enzyme inactivation process. At the lowest C_{out} (0.29 μM), around 70% of the drug was unaccounted for at steady state, suggesting the presence of other unidentified pathways of metabolism. These pathways were probably saturable because material balance (MB) increased from ~30% to ~77% as C_{out} values increased from 0.29 μM to 20.23 μM (Figure 3.15). These results show that metabolic saturation of unidentified pathway plays a key role in the dose-dependent reduction of E values. NOR and D-617 were the primary N-dealkylated metabolites observed in this study. Both metabolic pathways were nonlinear (Figure 3.13 and 3.14) and amounted to 50% - 30% of the total MB as C_{out} values increased from 0.29 μM to 5.77 μM, suggesting that metabolic saturation of N-dealkylation also contributed to the reduction of E values. The two phases of the NOR formation curve indicate that NOR formation was mediated by at least two enzymes in rats, and one of them was saturable at lower concentrations.

High infusion rates were used in our experiment to fully characterize the elimination profile of VER. The steady-state elimination rates of VER enantiomers exhibited a simultaneous first-order and capacity-limited extraction process within the concentration range studied, suggesting two or more enzymes were involved in the elimination of these two enantiomers. At least one has a low affinity and high capacity, and the others have a high affinity and low capacity. These results agreed with the findings by Gupta *et al.* (*1996*), which revealed a simultaneous first-order and capacity-limited elimination kinetics of VER in humans. Consistent with the results from Mehvar *et al.* (*1995*), S-VER was metabolized faster than R-VER in rats at a low infusion concentration (C_{in}: 2.24 μM, Table 3.11). However, it was found in our study that the stereoselectivity was quickly reversed and the difference was lost as infusion concentration increased. Based on the estimated parameters, the K_m values of the high affinity enzyme site(s) are 0.082 and 0.156 μM for S-VER and R-VER respectively, indicating that S-VER would reach saturation faster than R-VER. When C_{out} is higher than K_m of S-VER, R/S ratio would decrease and this ratio would increase as C_{out} exceeds the K_m of R-VER. Once saturable concentrations for both enantiomers are reached the linear processes would dominate. At these concentrations, R/S ratios approach 1 because clearance values of the two enantiomers are similar (CL_2: S-VER: 1.8 ml/min/g liver, R-VER: 1.5 ml/min/g liver). In humans, a sustained-release dosage yields R/S ratio higher than that of an immediate release form (*Longstreth 1993, Karim and Piergies 1995*). However, the lowest R/S ratios were also observed with low dose (at the beginning and at the end of each dosing interval) (*Longstreth 1993*). Little information has been published that allows careful examination of how sensitive the R/S ratio might be to small changes in the release characteristics of an oral formulation. The mechanism(s) responsible for this fluctuation in the R/S ratio over the dosing interval at a lower dose have not yet been identified. Nevertheless, the pooled steady-state Michaelis-Menten parameters obtained from the rat are very comparable to that of the human (V_{max}: 575 mg/day and K_m 0.292 μM for racemic VER) (*Wagner 1984*), indicating that saturable processes occur in the clinically relevant dose and concentration ranges. In addition, the high sensitivity of VER saturation to the input rate in low dose range might contribute to the considerable intra-

and inter- subject variation after oral VER administration (*Eichelbaum and Somogyi 1984*).

4.3.2 Enzyme inactivation of verapamil

Enzyme inactivation was found to be unrelated to the major N-dealkylation pathways. A characteristic "hump" suggests that the enzyme responsible for the formation of D-620, a secondary metabolite of NOR, was inactivated. This "hump" was observed in LID (*Tam et al. 1987*) and DZ (*Hussain et al. 1994*) metabolism that involved N-dealkylation. However, D-620 was a minor metabolite in the rat liver, the probable contribution of enzyme inactivation to the time-dependent kinetics in this study was not likely to be high. Further supporting evidence was provided from the pretreatment study. It is postulated that multiple administration of VER would be adequate to cause a marked reduction in enzyme activity, and the time-dependency would be diminished. If enzyme inactivation occurred, we would expect a significant reduction in T_{ss} for VER pretreated group. Our results failed to reveal any significant changes in the kinetic parameters, such as steady-state extraction, R/S ratio, and T_{ss} except for the formation of D-620. There are several possible explanations for the lack of drug pretreatment effect on the liver perfusion study. First, it is possible that the vulnerable isozyme was resynthesized during the 24-h dosing interval. However, this postulation is unlikely since the turnovers half-lives of CYP heme and apoprotein moieties range from 7 to 19 and 10 to 20 h respectively (*Correia 1991 and Gasser et al. 1982*). Evidence in the literature has shown that 31% of total CYP remained complexed for 24 h after a single treatment of rats with erythralosamine (*Delaforge et al. 1983*). Using kinetic analysis, Saville *et al*. (*1989*) estimated that metabolic activities of CYP were impaired for about 35 days following single dose pretreatment of LID. The estimated half-life for enzyme turnover was 25 days, as computed based on the rate of enzyme regeneration of 0.026 day^{-1}. Another possible reason is a lack of metabolic intermediate complex formation *in vivo*. The absence of metabolic intermediate complex formation for VER has been observed by Renton (*1985*) in Swiss train mice, even though it was demonstrated that the CYP dependent N-demethylation of aminopyrine N-demethylase was competitively inhibited by VER.

This unaltered perfusion kinetics after VER pretreatment seems to be inconsistent with the results from a previous *in vivo* study, which has shown an altered kinetics after multiple dosing (Table 3.6). Contrary to this pretreatment study (dose interval 24-h), a more frequent dosage regimen was applied in the *in vivo* rat study (dose interval 8-h). More frequent dosing could cause a significant accumulation of VER in the liver tissue, which provided a "sink" for the drug and a "source" of the drug for metabolic enzymes and caused the non-linear kinetics of VER.

4.3.3 Liver tissue accumulation of verapamil

The time to reach steady-state VER concentration in the liver effluent was unexpectedly long (Figure 3.18). Assuming that the liver is a well-mixed compartment, the mean residence time of perfusate in the liver is between 20-60 seconds dependent on the medium and flow rate (*Gray et al. 1987*). This time is much shorter than the time for VER to achieve a steady state for VER in this study. One explanation is that the drug may have a high affinity for the cellular region of the liver, which can act as a large reservoir for drugs. During initial drug administration, it can take a long time to fill the reservoir (*Saville et al. 1992*). The total amounts of VER bound and/or distributed in liver tissue were 594 ± 26.9 and 652 ± 26.1 nmol/g of liver for S- and R-VER respectively at C_{in} of 43.3 ± 3.0 μM (Table 3.13). These values could represent the total accumulation (binding and partitioning) capacity of VER enantiomers to the liver tissue, since the mean material balance of VER reached a plateau at this input concentration (corresponding mean C_{out}: 21 μM, Figure 3.18). These amounts accounted for about 40% of the infused VER between time 0 to steady state at a C_{in} of 43.3 ± 3.0 μM. These results clearly demonstrate that the accumulation of this drug in liver tissue was extensive. The percentage of tissue accumulation would be higher with lower input rate. For example, the total accumulation capacity would be 2-3 times higher than the total amount of VER administered for rats that received 10-mg/kg oral dose. Extensive tissue binding has been found to contribute to the time-dependent kinetics of DZ (*Hussain 1994*).

The high affinity of VER and NOR for liver tissue was confirmed by the stop infusion study as denoted by high K_{tw}s and long washout half lives (Table 3.13). Detectable levels of VER and NOR were found in the effluent at least 60 min after cessation of drug infusion. If there were to be no binding, it is expected that the organ should be free of drug within 2-3 min (*Saville 1989*). The binding of VER is stronger and more extensive than DZ, based on a comparison of the washout half-life ($T_{1/2}$) ~15 min for VER *vs.* ~1.82 min for DZ and K_{tw} ~50 for VER *vs.* ~8 for DZ (*Hussain et al. 1994*). The feature of the effluent time course for VER and NOR provides evidence for both partitioning and/or binding (Fig 3.18). The partitioning process may be represented by the initial first-order decline, as observed by Saville *et al.* (*1992*) for lidocaine. The slow release of bound drug and metabolites seems to be an another factor leading to a slow rise of VER level to steady state, because of the long equilibration time between the unbound species in the liver and the outlet perfusate. It is interesting to note that both NOR enantiomers have lower K_{tw} values but higher $T_{1/2}$ values, indicating a longer retention of NOR in the liver tissue. No stereoselective accumulation in and release from the liver were observed for either VER or NOR. Thus, hepatic tissue binding could not be the sole explanation for time-dependent reduction in E.

There was a characteristic sigmoidal rise of VER concentration with time at a C_{in} of 2.2 μM VER (Figure 3.10) and this feature disappeared when VER was infused at higher concentrations (Figure 3.9 and Figure 3.18). Furthermore, the time to achieve steady state decreased at higher C_{in} of VER. These observations are consistent with the characteristics of saturable binding to the liver tissue (*Gray et al. 1987*).

4.4 Isolated rat hepatocyte studies

In this study, the ability of several tertiary amines to inhibit the *in vitro* metabolism of VER enantiomers was examined. The results from these *in vitro* experiments indicate that the disappearance rate of VER enantiomers was inhibited by these tertiary amines in a dose dependent manner. Uptake study of VER indicates that a non-stereoselective linear uptake process dominated within the concentration range studied and the uptake of VER was not affected by the tertiary amines. Therefore, the inhibition of the disappearance rate of VER by these tertiary amines was not due to the decreased availability of the substrate to hepatocytes; rather, the inhibition was mediated through an interaction at the level of CYP isozymes or at intracellular binding site(s).

However, one should be cautious in extrapolating these results to intact animals. One of the limitations of *in vitro* approaches is the need to utilize substrate concentrations that are one or more orders of magnitude higher than those encountered clinically. *In* *vitro* studies of high substrate concentrations can be extrapolated down to a clinically relevant concentration range as long as mathematical models remain valid over the entire range (*Schmider et al. 1996*). However, a "high-affinity" metabolic reaction that contributes importantly to a drug's biotransformation at clinically relevant concentrations could be overlooked or underestimated *in vitro* if assay sensitivity limits the use of low substrate concentrations. Contrary to our liver perfusion study, no difference in pooled Michaelis-Menten parameters between S-VER and R-VER could be detected in this freshly isolated hepatocyte system (Table 3.14). This discrepancy may be partly due to the fact that a high substrate concentration range was used (5 to 50 μM of VER). At these concentrations, the stereoselective high affinity and low capacity enzyme sites would be saturated. According to the liver perfusion study, K_m values of these sites are 0.082 μM for S-VER and 0.156 μM for R-VER. The Michaelis-Menten parameters measured in this study reveal only the low affinity and high capacity enzyme sites, which are non-stereoselective.

4.4.1 Inhibition effects of tertiary amines — coincubation

Competitive inhibition between VER and the three tertiary amines was observed, suggesting VER shares the active sites on same isozymes for metabolism. The inhibition by DZ and DPH was noncompetitive. The mixed type of inhibition of both tertiary amines implies that they may also bind to the enzyme at a site other than the active sites and as a result causes a change in the structure of the enzyme, especially the active site (*Campbell 1995*). In rats, multiforms of CYP are capable of mediating the metabolism of LID, including CYP2C11, CYP3A, CYP1A2 and CYP2B1 (*Smith et al. 1991*). Unfortunately, information regarding particular isozymes responsible for DZ, DPH, and

VER metabolic pathways in rats is lacking. The metabolic rate of VER was reduced, but the effect on rate of NOR formation was different: decreased by DZ, increased by DPH and unaltered by LID (Figure 3.28). These results implied that a multienzyme system contributes to the metabolic clearance of VER and the inhibition of VER metabolism by these tertiary amines was mediated through an interaction with different isozymes.

The therapeutic consequence of competitive drug interactions is dependent on the relative affinity of the interacting drug for the catalyzing enzyme. The fact that K_i values for each of these tertiary amines are higher than the K_m values of VER suggests that VER has a higher affinity for the enzymes, and that there is little chance for these tertiary amines to effectively compete with VER for the active site of metabolic enzymes (Table 3.14). It is usually appropriate to use the unbound concentrations for prediction of the drug and inhibitor concentrations at the site of enzyme, since it is generally felt that this value reflects the concentration which is available to interact with the enzyme (*Bertz and Granneman 1997*). However, total plasma concentrations of the inhibitors were used as conservative estimates of the concentrations of the inhibitors in this study. According to this prediction, simultaneous administration of any of these tertiary amines with VER may result in insignificant pharmacokinetic interaction as predicted by the hepatocyte system.

4.4.2 Inhibition effects of tertiary amines — preincubation

Interestingly, our pretreatment study showed that LID, DZ and DPH were mechanism-based inhibitors, since the inhibitory effects were significantly enhanced by pre-treatment of hepatocytes with these tertiary amines prior to the addition of VER. These *in vitro* results support the contention that inhibitory metabolites may be generated during the pre-treatment and the inhibition by LID, DZ and DPH is mechanism-based. The generation of these metabolites is a potential source for interaction between these drugs and VER during prolonged therapy. Impairment of *in vivo* clearance by mechanism-based inhibitors such as erythromycin (*Wrighton et al.* 1994, *Olkkola et al. 1993*) and cimetidine (*Serlin et al. 1980, Ioannoni et al. 1986*) were substantially greater than those predicted from *in vitro* co-incubation studies, which provided high K_i values. Study in both rabbits and human treated with erythromycin for 10 days demonstrated that the metabolism of theophylline was impaired but this did not occur with short dosage periods (*Hemsworth et al. 1981*). NOR formation rate was reduced to a larger extent when the hepatocytes were preincubated with LID and DZ, indicating that a mechanism-based inhibition was involved in the N-dealkylation pathway. Although there was no direct evidence of MI formation for LID and DZ, both drugs showed time-dependent enzyme inactivation, which was related to the N-dealkylation pathway in rats (*Tam et al. 1989, Hussain et al. 1994, Murray et al. 1997*). It has been shown that DPH has the ability to inactivate some P450 isozymes *via* MI formation (*Bast et al. 1990*) and this inactivation is related to CYP2B1/2 (*Murray et al. 1992*) in the rat. In this study, DPH did not cause a decrease in NOR formation in both coincubation and preincubation systems, suggesting CYP2B1/2 were not involved in the N-dealkylation of VER in rats.

4.4.3 Product inhibition

Results of current hepatocyte study are consistent with the hypothesis that NOR can inhibit parent drug biotransformation. It was found that the inhibition by NOR was competitive and stereoselective, favoring the inhibition of the S- rather than the R-enantiomer (Table 3.14). However, the inhibition constants (K_i's) of NOR enantiomers were more than two times higher than K_m's of VER enantiomers, and the magnitude of the interactions was small even if the concentration of NOR was equal to the concentrations of VER at the enzyme site. Furthermore, NOR did not show evidence of being a mechanism-base inhibitor (Table 3.15). Thus, according to the prediction by this *in vitro* system, competitive inhibition couldn't account for the time-dependence of VER observed in rats.

5. SUMMARY AND CONCLUSION

Compared with single-dose oral administration, a significant increase in bioavailability and a significant decrease in oral clearance have been reported after multiple oral VER administration to humans. Although the pharmacokinetics of VER has been investigated extensively in both humans and rats, the mechanisms responsible for the changes in the kinetic behavior of this well-studied drug are highly controversial. In the current study, the underlying mechanisms were studied by evaluating the alterations of VER kinetics in relationship to input rates, dose and concomitantly administrated drugs. Appropriate *in vitro* and *in vivo* models were used to achieve these goals.

An in-depth analytical study was conducted to investigate the apparent pH dependent fluorescent response. Results showed that the fluorescent intensity and extraction efficiency of VER decreased to approximately half when the solution pH was increased from 2.2 to 8.0. This pH dependency can be alleviated with Tween 20 and the effect was totally abolished with BSA. These characteristics suggest that VER is surface active. Interfacial tension experiments using axisymmetric drop shape analysis confirmed that VER was surface active. Using labeled VER, it was discovered that the reduction of VER response was due to the adsorption of VER onto pipette tips during sample processing. This study indicates that the quantification of VER in aqueous solutions requires caution. It is recommended that pipette tips that do not adsorb VER be used. Otherwise, individual standard curves should be constructed for the quantification of VER at a specific pH to avoid errors. Addition of bovine serum albumin to aqueous solutions of VER can also be used to eliminate adsorption problem.

In Sprague-Dawley rats, time-dependent kinetics of VER was demonstrated by comparing single and multiple dose kinetics. After five VER oral administrations (10 mg/kg every 8 hours) a 36.8% increase in AUC, a 53.7% increase in oral bioavailability and a 29.9% decrease in oral clearance of S-VER were observed. At the same time, the kinetic parameters for R-VER did not change significantly. The AUC of NOR and NOR/VER ratio after multiple dosing were significantly increased for both S-NOR and R-NOR, indicating an accumulation of NOR.

The mechanisms of time-dependent kinetics of VER were further investigated using a single-pass isolated perfused rat liver system. The elimination of VER followed a combination of Michaelis-Menten and first-order kinetics within the input concentration range studied (2.2-43.3 μM). Since the degree of saturable liver metabolism differed for the two VER enantiomers, with S-VER reaching saturation faster than that of R-VER, the extraction of the drug was stereoselective and concentration dependent. It was found that VER was extensively accumulated and strongly retained in liver tissue as denoted by a high binding capacity (S-VER *vs.* R-VER: 594 ± 26.9 *vs.* 652 ± 26.1 nmol/g liver) and long washout half-lives (~15 min). This was also reflected by the partition coefficient of VER in the liver tissue *vs.* buffer solution in the sinusoid (S-VER *vs.* R-VER: 70.9 ± 3.0 *vs.* 73.9 ± 4.0), indicating that the liver tissue contained approximately seventy times higher concentrations of VER than did the corresponding sinusoid at steady state. There was a characteristic sigmoidal rise of VER concentration with time at low C_{in} of VER and this feature disappeared when VER was infused at a much higher concentration. Furthermore, the time to achieve steady state decreased at higher C_{in} of VER. These observations are consistent with saturable binding of VER to the liver tissue. The portion of drug that is bound to liver tissue, to some extent, determines the concentration of drug that is available for metabolism. At low doses, binding plays the most important role in the "apparent elimination" of VER. The reason why stereoselective metabolism was observed in the *in vivo* studies was probably due to a slow release of VER from its binding sites.

The role of enzyme inactivation in the time-dependent kinetics was evaluated by pretreating rats with racemic VER. Pretreatment of rats with VER for 5 days (10 mg/kg once a day) did not change the kinetic profile of VER in perfused livers. However, consistent with the *in vivo* findings, the metabolism of NOR was found to be impaired. This change can be attributed to the inactivation of enzyme(s) involved in the N-dealkylation of NOR. The disappearance of the characteristic "hump" in the D-620 profile after VER pretreatment is a clear indication of this observation. The role of enzyme inactivation however was trivial because D-620 was a minor metabolite in the rat liver. Using isolated hepatocyte preparation, it was found that NOR was able to competitively inhibit the elimination of VER. However, the affinity of this metabolite to metabolic enzyme(s) was lower than that of its parent drug (Km/Ki < 0.4); it is unlikely that product inhibition by NOR is an important factor for the accumulation of VER during chronic dosing.

The effect of LID, DZ and DPH on the kinetics of VER using freshly prepared isolated rat hepatocytes was studied. It was found that all of these tertiary amines were capable of interacting with VER when each was co-incubated with VER, suggesting that each of them share common isozymes with VER in their disposition. Lineweaver-Burke plots indicated that the interaction with LID was competitive in nature and the interaction with DZ and DPH was of the mixed type. Uptake studies showed that the inhibition of VER disposition by these tertiary amines was not related to the decreased accessibility of the drug to the active enzyme site(s). Since the apparent K_i values of these inhibitors are relatively high compared to their therapeutic concentrations and the K_m values of VER, it is predicted that LD, DZ and DPH would have little effect on the hepatic metabolism of VER when they are administered together *in vivo*. However, prolonging their exposure time to hepatocytes significantly enhanced the degree of inhibition by these tertiary amines. These results implied that the inhibition by LID, DZ and DPH was likely to be mechanism-based inactivation and prolonged exposure of these tertiary amines might promote interaction with VER.

This *in vitro* study demonstrated that different prediction might be obtained dependenting on the design of the *in vitro* experiment. Although, the most facile predictions of drug interactions are based on the assumption of competitive inhibition, predictability of *in vivo* inhibition is reduced when drug interactions involve mechanism-based inhibition. It is very important to identify the existence of such a mechanism involved in drug interactions.

In summery, a rapid, pH dependent adsorption of VER to pipette tips needs to be accounted for during its quantification. A time-dependent reduction in oral clearance of VER has been observed in Sprague-Dawley rats. The time-dependent kinetics in rats appear to be mainly due to an increase in the bioavailability of S-VER, which is a result of saturable liver metabolism and extensive but saturable hepatic tissue binding. Conversely, enzyme inactivation and product inhibition by NOR probably play a minor role. LID, DZ and DPH can alter the metabolism of VER through both competitive (and noncompetitive for DZ and DPH) and mechanism-based enzyme inhibitions.

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